

Peace dividend still in escrow

The welcome reduction of US military expenditure in this week's budget will not be enough to satisfy the Congress, but more can be had only by pushing the administration to accelerate the arms control process.

PRESIDENT George Bush owes more to his counterpart in the Soviet Union than he is allowing. The cash saving of \$6,000 million in the military budget of the United States between the current financial year and that beginning in October will, by itself, help ease the painful and protracted transition to a balanced budget, but it is only a small part of the potential saving that lies tantalizingly ahead. Were it not for the changes brought about in Eastern Europe last year, the Pentagon would have been wanting to spend more next year, not less. But the administration's view that there has not yet been time for the radical reappraisal of the defensive posture of the United States that new circumstances dictate is altogether too cautious. It may even be dangerous.

On present spending plans, the United States will continue for the two years ahead to be a strategic power in the 1980s mould. Procurement of the MX missile and development of the Midgetman missile will continue despite the prospect that, half way through this year, there will be an agreement to reduce strategic missile strengths by a half. Knowing that, the Congress will have its knife into those parts of the budget in the months ahead. The administration will protest that neither the changes in Eastern Europe nor even those in the Soviet Union mean that the need for strategic preparedness has disappeared. That is correct. What has changed is the nature of the strategic threat. Would it not be prudent that arrangements for strategic preparedness should themselves be transformed? And quickly?

Mr Gorbachev's achievement in the past several months has been to demonstrate to all concerned that the Soviet Union's enemies are internal. The Eastern European states have seized a measure of independence, while the fissiparous and not always wholesome nationalistic tendencies of the Soviet republics are plainly an embarrassment to Moscow, not least because the central government seems unable to decide how best to cope with them. Meanwhile, it is plain for everybody to see that the Soviet civil economy is nearly bankrupt, while the cascade of domestic elections due to begin next month will surpass last year's embarrassing dose of defeat for the Party's discredited officials and placemen. The Soviet military establishment remains in being (shorn of some 500,000 personnel), of course, together with its equipment, but what kind of threat does it pose for the United States?

The worst, if almost unbelievable, case is that there will

be an attempt to roll back the clock. On one view of recent events in Eastern Europe, what has happened there is merely that the ruling Party has created the illusion of popular revolution so as to mollify public discontent. On a bizarre extension of the same conspiracy theory, the Soviet party apparatus foments discontent in the republics as a means by which growing chaos can create a need for martial law, the postponement of next month's elections and a return to the bad old days, with the same boys in the jobs to which Brezhnev appointed them. Then, the argument goes, the Cold War can be declared afresh and we shall all return to the familiar 1970s.

This argument makes no sense. It is contradicted by the plain truth apparent on every television screen for the past several months that the upheaval in Eastern Europe and the Soviet Union has been sustained by people's wish to live differently — to be more prosperous and to be less often pushed around — and has been made possible by officials' often uncomprehending search for ways of meeting these wishes without too much discomfort for themselves. The process would have been faster if the people had a better understanding of the democratic process and if officials had more often been able to grasp the rudiments of the industrial economy.

The realistic worst case, for the months and years ahead, is that the hunt for new ways of choosing governments and new ways of arranging to turn raw materials into goods that people will buy will continue indefinitely. The military danger, then, would be chiefly that regional conflicts within Eastern Europe might spread towards the West. The federal republic, for example, might feel engaged by a resurgent Poland's claim on territory in East Germany, while Turkey (or Iran) might regard a reassertion of centralism in Moscow as an occasion to make common cause with one of the southern republics recently at each other's throats. But these are not strategic threats. There, the worst case is that the Soviet Union might choose to renew the Cold War, denying itself the technological and financial assistance needed to make good seventy years of neglect. Neither threat is credible. Both can be diminished to vanishing point — the first by reaffirmation of the Helsinki final act, with its guarantees of existing frontiers, the second by creating more durable mechanisms for helping the East trade its way out of its accumulated difficulties. Mr Bush will have his say at the conference at Vienna to be held in June.



On past form, the budget process will begin in earnest only about then. In the meantime, there is much useful work that can be done. Strategic weapons have no doubt come to stay, but their putative uses are different, more like those of the 1960s than the 1980s. A residual capacity to deliver devastating retaliation should suffice for any superpower's peace of mind, while sophisticated battlefield uses for nuclear weapons have been made positively dangerous by the assertion of independence by Eastern European states. That is why the strategic missile negotiations should now be aimed at lower limits — who needs 5,000 warheads in present circumstances? — while a nuclear-free zone (however narrow) in central Europe has become an urgent need. That is where the US Congress should be looking for relief from the pressures of the military budget.

In the not very long run, it should be possible to dispense with either MX or Midgetman (or even both, because there will still be submarines afloat). Meanwhile, the balance of US strategic interests (like that of the Soviet Union) will be turned from planning for major conflicts to the need to safeguard regional interests whose military connotations are very different. That is where the US Congress should be looking for relief from the pressures of the military budget. □

Scandal upon scandal

Chandigarh University has been complacent about the controversy over Professor V.J. Gupta's Himalayan fossils.

THE article by John Talent on page 405 of this issue is the fifth in a line of long exchanges between the principal disputants in the case of the peripatetic Himalayan fossils, which erupted in this journal last April. (The controversy goes back to 1981, but was marked by a public row at a symposium in Canada in 1987 and by a publication by Talent and three colleagues the following year.) The central figure is Professor Vishwa Jit Gupta of the Punjab (or Panjab) University at Chandigarh, who is accused of deceiving several co-authors in India and elsewhere by falsely attributing, to poorly defined sites in the Himalayas, fossils whose provenance is unknown ("salting") and by using the same fossil specimens in research articles describing finds at different places ("recycling"). What is the non-palaeontologist to make of these exchanges?

The debate hinges on obscure details of Himalayan palaeontology and stratigraphy made no easier by variations in the rendering of place-names where most people never go (and often cannot go because of military security). But close reading of the accusations and the responses leaves the impression that Gupta's defence is flimsy; he has not dealt specifically with issues where the arguments on one side and the other are open to independent verification. That he should have reacted with anger to Talent's first accusations is understandable, but even his more temperate response last week (*Nature* 343, 307;

1990) to his erstwhile co-authors consists largely of the assertion that they are mistaken or misguided. In prudence, Gupta might also have made some of the disputed fossils available for isotope examination by others, so that their provenance might be checked. (At an early stage, he declined an offer to help arrange such an examination on the grounds that he hoped soon to acquire the necessary equipment.) The result is that most of the puzzling anomalies to which Talent originally drew attention remain unexplained.

What will happen next? Palaeontologists are by now sufficiently well seized of the problem of the Himalayan literature to know when judgement on particular research reports should be suspended. The *Journal of the Geological Society of India* published last December an article by John Talent and associates together with a leading article headed "Indian palaeontology under a cloud". The Pander Society, an informal body of people with a special interest in conodonts, has advised its members to discount conodont papers in which Gupta has had a hand and has drummed him out of membership. But it is important that Gupta is not friendless in the dispute; last week, J. B. Waterhouse, a long-standing collaborator of Gupta, chided Talent for his prose (too colourful), for exaggeration (which is conceivable) and for having raised serious accusations without "serious documented research" (which is belied by the weight of evidence now brought to bear). The truth is that there is a solid case to answer, and that Gupta has not answered it.

So what should be done? The greatest disappointment of the past few months is that so little seems to have happened in India to get to the bottom of the Gupta affair. There are some in India who cried "Prejudice!" when Talent's first article appeared, but that is an outrageous charge — and no defence. A. S. Paintal's Independent Society for Scientific Values and the Indian National Science Academy have separately mounted inquiries, but have little progress to report. Yet the prime responsibility rests with the university at Chandigarh, the institution most directly threatened by the accusations that one of its senior members is a fraud. Painful experience elsewhere has shown that a university thus maligned is well advised to mount an independent enquiry into accusations against its members. If Harvard University takes that line, can Chandigarh be wise to hang behind and risk being damned itself? Instead, the university is talking of a fact-finding expedition to the Himalayas, but named outside participants have not yet been invited.

The Gupta business raises a further difficulty. Three of those who have openly criticized Gupta's work are colleagues or associates of his at Chandigarh, and are now in an uncomfortable position. Whistle-blowing is always a dangerous trade, the more so when directed against more powerful colleagues. *Nature* is not in a position to offer protection of any kind to those concerned, but it is in a position vigilantly to report what happens to them in the months ahead. It will be a shame on India if that news is bad. □

Strong support for science in US budget

■ Big projects win backing

■ Twelve per cent rise planned

Washington

SCIENCE and technology fares remarkably well in US President George Bush's budget request for 1991, presented to Congress earlier this week. Although spending is set to rise by only 3 per cent in the budget as a whole, the president proposes a 12 per cent rise in civilian research and development and an 8 per cent overall rise in funding for basic research.

Increased support is pledged for almost all the 'big science' projects, including the Superconducting Super Collider and the Global Change programme. According to D. Allan Bromley, science adviser to the president, small science will not be cut as a result — he says he has been "very vigilant" to be sure that no harm comes to the programmes supporting individual researchers.

All this may seem too good to be true — and indeed it may turn out to be. Although Bromley says he is "delighted" with the 1991 budget, it is a fact that the request is not much larger, after correction for inflation, than that of the previous year. After the demands of Congress and the Gramm-Rudman Act were satisfied, real increases for science programmes in 1990 turned out to be considerably smaller than those requested by the president.

But despite the inevitable uncertainties, a clear trend is a rise in support for civilian research relative to military research. Changes in the Eastern bloc will help that trend along, although Bromley warns that basic defence research spending should be maintained in order to protect the United States from "technological surprises". The administration has not lost faith in the Strategic Defense Initiative (SDI) and an increase is requested from the \$3,800 million granted in 1990 to \$4,700 million.

The ending of the Cold War has been traumatic for the Department of Energy (DoE), which now faces both a legacy of radioactive waste accumulated from 40 years of bomb-making and a rapidly changing *raison d'être*. In the 1991 budget, weapons production and research grow to \$8,506 million (up \$843 million) but clean-up funding grows proportionately even more. \$2,791 million is requested for waste management and environmental restoration, an increase of \$573 million. Plutonium production is being largely phased out. In the absence of a need for new bombs, plutonium is needed only to supply the National Aeronautics and Space Administration (NASA) for space-borne power supplies.

In support of its science programmes, DoE proposes an increase of \$180 million, to a 1991 total of \$1,273 million. But fusion research is put on ice, at least temporarily. Until an independent policy review is completed on the wisdom of current magnetic-fusion approaches, DoE will not go ahead on the construction of the Compact Ignition Tokamak.

In keeping with Bush's promise to be the "environment president", the budget asks for a massive increase — from \$659 million to \$1,034 million — for the inter-agency Global Change Research Program. A 35 per cent increase goes to NASA, whose new Earth Observing System (EOS) is set for \$134 million. The National Oceanic and Atmospheric Administration is scheduled for a 383 per cent increase to \$87 million. The money is for increased observation, both on the Earth and above it, and new work on climate modelling.

Support for the Superconducting Super Collider (SSC) is less certain. For 1991, DoE has asked for only \$318 million, \$93 million more than received in 1990, but \$75 million less than originally planned. Although SSC's timetable says that construction crews should already have begun tunnelling, acting Office of Energy Research director James Decker says no real construction will take place this year or next. Bromley says that the project will focus on "getting reliable industrially producible magnets" this year. Magnet problems have caused cost estimates for the project to rise. If the increases become "excessive", Bromley notes that Congress may consider support for the project "open for discussion". Among the 'big science' programmes, the Human Genome Project has proved one of the most popular with Congress. The 1991 budget asks for \$154 million, with \$108 million coming from the National Institutes of Health (NIH) and the balance from DoE. Although that figure is over \$70 million more than received in 1990, it is substantially less than the \$200 million that project planners have been anticipating recently. The cut appears to mean that although the project is worthy, it may not be able to come up to speed as quickly as originally hoped.

NASA does well in the budget request, overall up 19 per cent over last year. NASA Administrator Richard Truly acknowledges "strong support" from Vice-President Dan Quayle. With a total allowance of \$15,125 million, Truly

intends to turn some of the grand ideas of last year into bona fide programmes: the EOS and planning for manned flight to the Moon and Mars are among them.

The first small but expensive (\$1,270 million) step on the journey to Mars will be the 'Lunar Observer', a robotic probe that will be put into a polar orbit around the Moon, and an enhancement of the data-collection capability of the Mars Observer, which is scheduled for launch in 1992.

The slimmed-down Space Station Freedom will consume \$2,600 million this year to proceed even at the reduced pace announced by NASA last autumn. But despite its troubles, Freedom is still, in Truly's words, the 'cornerstone' of the US space programme in the 1990s, and 1991 should see a transition from design to construction.

In the life sciences, research into AIDS continues to remain a high priority, with the Department of Health and Human Services (HHS) asking for a \$109 million (7 per cent) increase in AIDS research spending. This brings total support throughout the public health service to \$1,700 million for 1991, with the emphasis on basic science research, risk assessment and prevention. The NIH would receive \$800 million in AIDS funding, a \$57 million increase.

The administration is seeking a 4.7 per cent increase in NIH's overall budget. The number of new grants (5,095) would rise almost 10 per cent over 1990. This brings the total to 20,439 research project grants for which HHS has requested \$4,425 million in funds.

For the National Science Foundation (NSF), Bush once again calls for a doubling of the budget by 1993, although prospects for that look increasingly dim. In 1990 the agency's research budget actually shrank in real dollars, and the president's 1991 request is for only a 14.4 per cent rise over that. Nevertheless, this could be the year in which the agency shows some real growth.

Funding for education fares best in NSF's 1991 request. Undergraduate support is up 48 per cent to \$134 million, followed by money for graduate, post-graduate, and professional education, which is to rise 29 per cent to \$164 million.

NSF facilities also do well. New programmes such as a gravitational wave detector, two 8-metre telescopes and a high-magnetic-field laboratory comprise part of the \$536 million the agency intends to spend on new facilities. Other increases include \$25 million for 8–12 new Science and Technology Centers.

NSF director Erich Bloch admits that construction comes at the expense of new research grants, but he says it is better for NSF to select new facilities than to have Congress force its choices upon the agency. □

Sagan appeals to world religious leaders

Boston

ASTRONOMER Carl Sagan and 22 other well-known researchers chose Moscow as the unlikely venue for an appeal to world religious leaders to join scientists in protecting the global environment. The appeal came at a recent conference on the environment and economic development which attracted over a thousand religious, political and scientific leaders from 83 nations.

Ironically, Sagan travelled to the officially atheist Soviet Union to announce "a religious as well as a scientific dimension" to the problems of global change. Even more remarkable, the conference was sponsored by both the USSR Academy of Sciences and the Russian Orthodox Church.

The appeal states that "efforts to safeguard and cherish the environment need to be infused with a vision of the sacred". Among those who have given their backing are physicist Hans Bethe, evolutionary biologist Stephen Jay Gould and former MIT president Jerome Weisner.

The appeal certainly reached a global audience. It, and other parts of the five-day conference, were the first ever to be televised with satellite time provided jointly by the East and West communications networks Intersputnik and Intelsat and reached an estimated audience of 2,000 million people in 129 countries. Later at the conference, more than one hundred religious leaders joined to hail the scientists' appeal as "a unique moment and opportunity in the relationship of science and religion".

Seth Shulman

■ Global change 'consensus', page 401.

COMPUTER CRIME

Cornell hacker is convicted but free

Washington

A PREVIOUSLY untried 1986 law aimed at the prosecution of computer criminals passed muster last week when former Cornell University graduate student Robert Morris Jr was convicted for writing and releasing a "virus" that crashed thousands of computers nationwide in 1988.

Morris has not yet been sentenced, but he is expected to get relatively light punishment. His virus did no permanent damage and his defence attorney described the program as a harmless experiment in testing computer security that went awry. But because Morris' program was clearly written for the purpose of illegally breaking into remote computers and was then released on a national network, federal prosecutors had little difficulty obtaining a conviction.

Cases of a program with less obvious malicious intent or a more modest scope may not be resolved as easily.

G. Christopher Anderson

US relaxes export curbs

Washington & Munich

IN what administration officials say could be the beginning of a substantial thaw in the restriction of high-technology export to former Soviet-bloc countries, the White House announced last week that it would relax rules on sales to Eastern European nations that have abandoned Communism and are liberalizing their economies.

Although the details of the new policy have not been released, they are believed to include approval of computers six times more powerful than currently allowed, sale of fibre-optic cables and sophisticated machine tools.

The decision comes in response to strong pressure from European allies to rethink export restrictions given the explosive restructuring of Eastern Europe. Since the beginning of the Cold War the US and its allies have withheld technology from Soviet-bloc countries in an attempt to maintain a technological and strategic edge in the West. The Paris-based Coordinating Committee for Multilateral Export Control (CoCom) maintains a list of restricted technologies and monitors sales.

"We now face a situation in which the political and military environment is changing", State Department spokeswoman Margaret Tutwiler said in a statement released last week to explain the Administration's shift in position. State Department officials may suggest further liberalizations in June at a major CoCom meeting in Paris, Administration officials said.

Industry sources say that the Administration plans to allow the sale of 32-bit computers, a class that includes the Intel

80386 chip that is now common in advanced personal computers. Current CoCom rules allow sale of 16-bit computers of the IBM PC-AT class. The relaxation is also expected to allow scientific equipment based on 32-bit technology. Certain medical diagnostic instruments such as nuclear magnetic resonance spectrometers contain 32-bit microprocessors, as do many other data-processing and analysis devices that are commonly available in the West.

Some European nations reacted sceptically to the US announcement.

In West Germany, officials suggested that the new proposal is more bark than bite. "This is the third or fourth time we've heard that the US wanted to modernize the CoCom list. We've learned to be realistic about these promises", says West German Economics Ministry spokesperson Dede Gaw.

Another source in the ministry says that it seems unrealistic to have different restrictions for the Soviets and other nations — that would force too much responsibility on the other nations to enforce the restrictions. And trying to block out the Soviet Union smacks of Cold War policies, the source says.

Eastern European countries, on the other hand, generally welcomed the US decision, but several said they had not received official word from the administration on what specific changes were planned. US Ambassador Allen Went is expected to make a formal presentation at the June CoCom meeting outlining the specific technologies that would be deregulated.

G. Christopher Anderson & Steve Dickman

FOSSIL REPTILE

'Lizzie' set to stay at home

London

THE earliest-known reptile may end up in Scotland after all, following a surprise announcement from the Museum für Naturkunde in Stuttgart, successful bidders for the £180,000-specimen (see *Nature* 343, 106; 11 January 1990). Museum Director Professor Bernhard Ziegler has agreed to delay the purchase of the 338-million-year-old fossil, affectionately known as "Lizzie", until after 31 July 1990. This will give the National Museums of Scotland time to match the purchase price.

The 10-inch fossil was discovered by professional collector Stan Wood at East Kirkton in Lothian, Scotland. His price — £207,000 (including value-added tax), exceeded the budgets of UK museums. But it became clear that the Stuttgart bid was, in part, an attempt to stop the fossil

going to a private collection where it would be inaccessible to researchers. Rupert Wild of the Stuttgart Museum admits that Lizzie's proper place is in Edinburgh, with the rest of the East Kirkton collection.

Lizzie will be on display at the Royal Museum of Scotland between 1 March and 27 May: the trustees of the National Museums of Scotland have made available £104,000 from the 1990-91 purchase grant, and the museums will launch an appeal for the remainder.

Although Lizzie was described in *Nature* last December (342, 676; 7 December 1989), the author, Timothy Smithson of the University of Newcastle, did not give her a formal zoological name. But arrangements can be made to name her after a sponsor.

Henry Gee

EAST/WEST COLLABORATION

IIASA regains US support

Munich

In keeping with changing East-West relations, the United States has promised to restore its support for the International Institute for Applied Systems Analysis (IIASA). In a letter from White House science adviser D. Allen Bromley dated 5 January, the US government promises to resume full cooperation with IIASA, from which it had withdrawn in 1982 following suspicions that the institute contained Soviet spies.

IIASA was established in 1972 as an international think-tank at Laxenburg, near Vienna, by the United States, the Soviet Union and ten other countries. The institute brings in researchers from all its member countries, now 16, to study global problems such as forest decline and transboundary pollution.

Bromley set two conditions for US government reentry into IIASA: that the institute focus more on environment and climate change, and that it consider changing its name to reflect the new programme. IIASA spokesman Jean-Guy Carrier said the conditions will be easy to meet and that IIASA researchers were overjoyed by the change of mind.

Former Secretary of State George Shultz said in 1984 that withdrawal from IIASA was motivated by a desire to invest more in bilateral, rather than multilateral, research. But the real reasons had more to do with concern that IIASA was being used for Soviet intelligence gathering.

Support from the National Science Foundation was resumed in 1987 but the US government remained unwilling to help. Instead, the American Academy of Arts and Sciences, IIASA's official US 'partner' organization, pieced together the US contribution with donations from charitable foundations.

Several US senators urged the administration to change its view, and there was a 'concerted effort' by academies of science in the Soviet Union and Eastern European countries to persuade the United States to renew its support for IIASA, said Carrier. If the turnabout had not come, ousting the United States from the organization was a "serious possibility", he said.

But the dramatic changes in Eastern Europe probably made the biggest difference in changing the US position. Carrier said that the reorganized Czech State Commission for Science, Technology and the Environment has invited IIASA to present its ideas on air and water pollution in Czechoslovakia in March. It is the first invitation to IIASA by that country in 16 years, he said. East Germany has also invited IIASA to present a news conference in East Berlin on acid rain in April, another first.

Steven Dickman

ENERGY:FRANCE

New drive on car pollution

Paris

FRENCH government and industry last week announced their intention to build an energy-efficient and non-polluting 'green' car.

The logic behind the announcement is simple: last year, transport consumed as much energy as French industry and now burns 60 per cent of the nation's oil imports. Thirty per cent of all carbon dioxide emissions, 70 per cent of carbon monoxide and 10 per cent of sulphur dioxide emissions come from transport. And the trend is increasing, despite a reduction in the size of engines since the 1970s petrol crisis. Over the past 15 years, the urban use of the motor car has increased by 60 per cent.

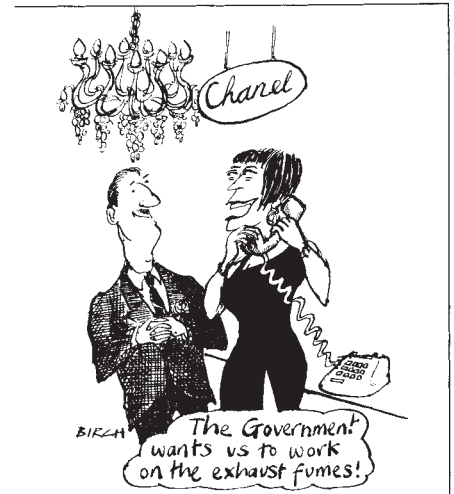
Backing for the green car comes in a joint protocol signed by three government ministries (research, industry and transport) and car manufacturers Peugeot SA and Renault. Over the next five years, the two companies promise to triple their research effort, while the government will encourage the state research organizations to play their part in "adapting the motor car to the environment".

Peugeot and Renault, usually thought of as competitors, will invest FF1,200 million (\$190 million) over the next five years in joint and separate ventures to make existing engines cleaner, while looking for new types of engine.

High on the list is the two-stroke, which produces few nitrogen oxides but is not very efficient. Gas turbines, possibly burning hydrogen fuels, will also be tried. Electric cars are already being produced,

but they have a daily maximum range of 100 km. To replace the small family petrol-engined car, they would need to have a range more like 500 km before recharging. Research is to be intensified into new kinds of battery. The manufacturers have given themselves five years for pilot studies and three to four years to build a prototype.

Some FF500 million will go towards



improving the energy-efficiency of existing cars, while reducing levels of pollution in line with European Communities regulations agreed last June. As more than half of all car journeys are less than three km — when engines are cold and therefore much less efficient — new engines are likely to incorporate some kind of pre-heating device.

Peter Coles

ENERGY:CALIFORNIA

Utilities see the green light

San Francisco

CALIFORNIA's electric-power companies are making friends with their long-time enemies in environmental and consumer-protection groups and discovering the surprising fact that there may be money in not generating electricity — or at least no more than is really needed.

In a new scheme proposed last week, cash rewards would be paid to consumers adopting energy-efficient technologies. The utilities see energy efficiency benefiting them principally because they will have to invest less capital in new power plants and purchase less electricity from alternate sources.

The plan brings together most of California's big electric-power companies, the Natural Resources Defense Council and the consumer organization Toward Utility Rate Normalization (TURN), as well as industry trade associations and government agencies.

In submitting the plan to the California

Public Utilities Commission (CPUC) for consideration, backers claimed that a \$500-million investment over the next two years would result in savings of more than \$1,000 million.

Rewards would go both to the small and the large: Pacific Gas and Electric, the largest US electric utility, talks of rebates of up to \$100 for the purchase of energy-efficient refrigerators and air conditioners, \$15,000 for commercial customers installing more efficient equipment and \$300,000 for large industrial customers building plants using the latest energy-saving technology.

Participants stress that the plan is not yet finalized. Each utility has outlined different means of achieving its energy-conservation goals and redistributing the cost savings to shareholders and rate-payers. By the end of March, CPUC is expected to establish several test studies to determine which are more efficient.

Robert Buderl

Reactor plans drawn up

Munich

NUCLEAR fusion scientists from the Soviet Union, Europe, Japan and the United States, meeting in Vienna, have come up with an approximate construction schedule and a cost for the International Thermonuclear Experimental Reactor (ITER), a machine intended to assess the engineering requirements of a full-scale fusion reactor. The estimated cost of building ITER is \$4,900 million, but for now the fusion community is asking the collaborating governments only for money to begin the design phase.

Exploratory planning has been going on since 1988 at Garching, West Germany, near the Max Planck Institute for Plasma Physics, and should be complete by the end of this year. According to the ITER council, which is administered by the International Atomic Energy Agency in Vienna, a detailed design could be ready by 1995, and the reactor itself would then take another six years to build. Although ITER is not expected to generate more energy than it consumes, it should demonstrate the feasibility of holding a burning plasma for a long enough time and in the quantity that a commercial reactor would need.

The final decision on whether to build ITER, which can come only after details of cost-sharing have been worked out and an acceptable site found, is not likely to be made before 1993. In the meantime there seems to be general enthusiasm for the engineering design phase. Europe would agree to begin the next phase, predicted West German fusion expert Siegfried von Krosigk of the Research Ministry. ITER would overlap by "98 per cent" with the Next European Torus (NET) that Europe is planning, he said, so collaboration would save money.

Von Krosigk said that the Soviet Union has "generally been very positive" in its attitude towards ITER. Japan is also likely to go along with ITER, hinted Ken Tomabechi, chairman of the ITER management committee. "I have heard nothing negative", he said.

The US fusion programme has still not recovered from the upsets of last year, when efforts were made to stall research into magnetic fusion to allow work on laser confinement fusion to catch up (see *Nature* 341, 476; 1989). The 1991 budget (see page 397) provides continued support for ITER, but does not provide money to begin building the next domestic reactor, the Compact Ignition Tokamak (CIT). If the political budget battles turn into a contest between ITER and CIT, money for both projects will become volatile.

Steven Dickman

Spaceport under a cloud

Sydney

US fears that the United States may lose technical information to the Soviets could put a stop to Australia's plans to build a 'spaceport' offering commercial launch services at Temple Bay on Cape York peninsula in far north Queensland.

The Australian plan calls for Soviet-made rockets to be used to launch satellites manufactured in the United States and other countries. The United States is likely to object on security grounds.

The Commonwealth government has endorsed the proposal by the Cape York Space Agency (CYSA) to build the facility, and negotiations to buy Soviet rockets, which are much cheaper than their US equivalents, have already been under way for two years. But in the second half of 1989, several pieces of legislation were put to the US Congress that could hinder the use of the Cape York facility by US manufacturers of satellites, according to Bruce Middleton, executive director of the Australian space office.

Only one of the bills has so far been enacted into law. An amendment to the Appropriation Bill for the Departments of Commerce, Justice and State, it prohibits the granting of export licences for the launch of US satellites on Soviet or Chinese rockets. The amendment is active only until October 1990.

A clause in the amendment provides US President George Bush with the power to lift the ban if it is in the national interest.

Last December, Bush used this clause to reinstate export licences, revoked in the

wake of the Tiananmen Square massacre, for two US-manufactured satellites purchased by Aussat Pty Ltd, an Australian company. They will be launched from a Chinese Long March Rocket in 1991.

Other legislation includes two amendments to the NASA Authorisation Bill. One prohibits the export of US-built satellites to other nations for launch on rockets from the Soviet Union. The other prohibits the use of Soviet rockets except in circumstances where the president certifies to Congress that the price of the Soviet launch "is not more than 25 per cent below the price of a comparable launch vehicle built in a market-based economy". Middleton says that the US legislation is being taken seriously in Australia, but "it is not an insurmountable obstacle and is capable of change". There are also problems back home. On 2 December, Queensland elected a Labor government which will back a study of the impact of the spaceport on the environment and on aboriginal heritage land. The proposed site, is considered traditional land by the Wuthathi Aboriginal Council.

The spaceport is to be a fully commercial venture but the Commonwealth support for the venture necessary to entice investors is conditional upon the satisfactory completion of environmental studies. "If at the end of the environmental impact study phase the Commonwealth and the State indicate that they are not in favour of the project then we will not proceed", says CYSA director Stephen Williams.

Tania Ewing

REMOTE SENSING

Successful launch for Spot-2 shop-window

Paris

AFTER a series of setbacks over the past several weeks, the French commercial remote-sensing satellite, Spot-2, was launched successfully by an Ariane 40 rocket on the night of 21 January. Accompanying Spot-2 into space was a payload of 'auxiliary passengers' — six microsatellites for British and US customers.

Spot-2 was built by the French company Matra for the state space research centre (CNES) and will enable its twin, Spot-1, to be retired at last. Spot-1 was launched in February 1986 with a planned lifetime of two years. Its continued operation, despite a failed tape recorder on board, has meant that CNES has been able to recoup development costs faster than expected.

But Spot Image SA, the company created by CNES to sell Spot pictures, is still far from making a profit. Last year, the company's turnover was FF130 million, while, in 1988, overheads were running at FF210 million. And Spot-2 is estimated to

have cost FF500 million to build.

Nevertheless, Spot is a good shop-window for French space technology. Next will come Spot-3, due for launch at the end of 1992. Last July, Prime Minister Michel Rocard gave the go-ahead to build Spot-4.

The Ariane launch also put six microsatellites into low orbit. Two of them (UOSAT D and E), weighing less than 50 kg, will be used to 'store and forward' messages from their owners, the University of Surrey in Britain, and more than 200 British schools and universities. The other four satellites each weigh only 12 kg and belong to the US amateur radio satellite corporation, AMSAT. They will be used for communications, education and colour photography using a charge-coupled device camera.

Although microsatellites are still uncommon in the West, the Soviet Union has been launching its Kosmos satellites, weighing 40–50 kg, since 1964.

Peter Coles

METEOROLOGY

Storm success came easy

London

THE UK Meteorological Office is this week congratulating itself for predicting successfully the hurricane-force gales which ravaged the British Isles on 25 January, leaving a trail of destruction and 47 dead. Strong winds were predicted when a deepening depression was picked up in the central Atlantic the previous weekend. When the pressure at the centre of the weather system began to plummet on the afternoon of 24 January, they forecast that a severe storm would hit the United Kingdom the next day, and warned that "structural damage is expected".

The Meteorological Office is keen to stress the accuracy and speed of its forecasts, following its failure to give similar warning of the storm that devastated southern England on 15–16 October 1987, claiming 19 lives. In 1987, the Meteorological Office's two forecasting models — a 'fine mesh' model, which gives detailed predictions, and a 'coarse mesh' model, which is less detailed

Not far from *Nature's* London office, a tree felled in the 25 January storm. (AP)

but is updated more often — gave different results. This time, the models agreed, says Martin Morris, head of central forecasting.

In the wake of the 1987 storm, a report commissioned by the Ministry of Defence, which funds the Meteorological Office, concluded that training was inadequate and that senior forecasters were not afforded sufficiently high status. Since then, training has been extended and career structure overhauled. Televised weather forecasts now include more explicit storm warnings, and the system of notifying the emergency services has been streamlined.

But the nature of the storm, rather than changes in the Meteorological Office, provides the main reason for the difference

in performance in 1987 and this year. Professor Brian Hoskins of the University of Reading explains that the 1987 storm did not have an obvious precursor in a marked depression, but was formed when several less obvious features came together. In its final approach to the English coast, the storm gained energy very rapidly as condensing rain released latent heat into the system. Morris says that the full potential of the 1987 storm

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

became apparent only a few hours before it came ashore.

This year's storm had a more leisurely and predictable development. Nevertheless, the occurrence of two hurricane-force storms over the English mainland within the space of three years raises the question of global climate change. Hoskins says that the storms were unusual, not in their intensity, but in their location. Usually, similar storms either 'blow themselves out' over the Atlantic, or pass over sparsely populated areas in northwest Scotland. He is currently examining the influence of greenhouse warming on storms, and believes that local shifts in storm paths are a possible consequence.

Peter Aldhous

CLIMATE CHANGE

New international projects planned

London

CLIMATOLOGISTS from Europe, the United States, Brazil and the Soviet Union meeting in Wallingford, Oxfordshire, earlier this week, agreed to increase the emphasis on long-term prediction of climate change in the International Geosphere Biosphere Project (IGBP) and the World Climate Research Programme (WCRP).

IGBP and WCRP are the two largest climate research programmes and the Wallingford meeting was of a joint committee aiming to coordinate efforts to improve the accuracy of general-circulation-model

(GCM) predictions.

IGBP has chiefly been interested in the climatic effects of vegetation and greenhouse gases, whilst WCRP has concentrated on the interaction between the Sun's energy and the Earth's surface, and its effects on water relations. Climate prediction using GCMs requires complementary data from all of these areas.

Major studies agreed by the IGBP-WCRP committee include those on desertification in the Niger Sahel, deforestation in the Amazon basin, and of carbon cycling in the Canadian boreal forest. Peter Aldhous

CLIMATE CHANGE

Towards a 2°C consensus

Washington

EARLY signs of a scientific consensus on global warming emerged last week as top-level representatives from more than 40 countries prepared to gather in Washington next week for a meeting of the Intergovernmental Panel on Climate Change (IPCC).

Briefing a National Academy of Sciences panel on global change, a panel of atmospheric scientists agreed that significant effects from the greenhouse effect may now be considered "very probable." Although there was some disagreement on the magnitude of the expected temperature rise, almost all of the scientists briefing the academy believe mean global temperatures will go up by at least 2 °C by the middle of the next century. But the rise will not be uniform — the latest models show that the Northern Hemisphere, with a large land mass, will warm far more than the largely oceanic Southern Hemisphere.

Although the United States is not expected to announce any radical policy changes at the IPCC meeting next week, President George Bush may use the opportunity to highlight environmental features, such as a new \$170 million tree-planting initiative, in his 1991 budget, which was released earlier this week (see p. 397). The administration is already touting a \$500 million increase in global warming research funding for 1990, but most of that rise is due more to creative accounting than new money. Real increases in 1990 amount to about \$60 million above 1989's \$134 million research budget.

The administration may have to get its own house in order before it can hope to take a leading role on international global warming policy. The US government continues to be characterized by "inter-agency squabbling", according to Alan Miller of the University of Maryland's Center for Global Change. But with several European countries beginning CO₂ emission reduction programmes, the US administration risks falling out of step with international opinion on global warming. For that reason, President Bush has called for a White House meeting of top-level scientific and economic advisers from selected countries in the Spring. The meeting will help the Administration "determine where the uncertainties are in the science and economics of the issue", a White House spokeswoman says.

Congressional action may be on a faster track. In both the House and Senate, part of major bills to promote energy efficiency, emission controls, and reforestation are expected to pass this year.

G. Christopher Anderson

Visa veto worries dissidents

Washington

REACTION among students from mainland China in the United States ranged from indignation to cautious optimism last week after Congress failed by four votes to overturn President George Bush's veto of a bill that would have allowed the students to stay in the United States — and avoid possible punishment on their return to China — for up to four years after their visas had expired.

The President argued that allowing the bill to become law would have further strained relations between the two countries and left the United States with far less leverage in ensuring China's compliance on human rights. In place of the vetoed legislation, Bush has promised to issue an executive order that would serve as a functional equivalent. This, he says, will be perceived as less antagonistic by the Chinese government. But some Chinese students say that they do not trust the administration to keep its word.

Yuan Liu of the Massachusetts-based China Information Center "fears for the future" of Chinese students in the United States, with nothing but the President's executive order protecting them from deportation. He says that the administration's actions in past months — two secret trips by top-level administration officials to meet with Chinese leaders, and a partial lifting of trade embargoes — have left the students with "a lack of confidence" in the administration's policy on China.

Liu argues that the only reason Chinese leaders may be less offended by an executive order, rather than congressional legislation, is that they recognize that an order can be lifted at will whereas a law can be removed only by an act of Congress.

Chinese students worry that policy could be reversed at some point in the future to appease China, he says. "The majority of students here would like to go back some time, but now is a bad time." Most Chinese students in the United States fear they will be labelled as dissidents if they return to China, Liu says.

Others in the US Chinese population greeted Bush's promise with more approval. "A law reflects some kind of permanency. But with China we're dealing with a changing situation", says one Chinese-American university professor. Bush stated last week that improved relations are necessary between the countries to encourage the continued exchange of students. Chinese officials have indicated that the future of the Fulbright Scholar exchange programmes, which sponsors Chinese students in the United States, might depend on Bush's veto of the congressional bill. China halted the Fulbright programme in retaliation for sanctions

imposed after the Beijing riots last summer.

There are 40,000 Chinese students in the United States, of which 32,000 hold visas requiring them to return to China before renewing or changing their status. Students holding such a visa, known as a 'J-1', must return to their country for at least two years before reapplying. The congressional bill would have given Chinese students up to four more years to apply for new visas or for permanent residence without returning to China.

Seven thousand students have been allowed into the United States since the Tiananmen Square massacre last June. Since the demonstrations, application to study in the US have risen more than 60

per cent. But more than half of those applying are rejected by the US Embassy because they cannot prove that they are well qualified for the programmes they have chosen, that they will be able to support themselves, or that they will return when their studies are over.

Most of the students and workers who participated in the June demonstrations are still at large, a situation the Chinese government is said to find embarrassing. Nevertheless, thousands of others have been arrested. At least 40 protesters have been executed since June, according to Asia Watch, a human-rights organization. Although most of those brought to trial have so far been workers and unemployed, Asia Watch reports that authorities have recently begun conducting secret trials of students as well.

G. Christopher Anderson

CLINICAL RESEARCH

Threat from UK reforms

London

THE British government's plans to reform the National Health Service (NHS) are coming under fire from the some of main bodies supporting medical research, for failing to account for the needs of clinical research.

The bill, now being discussed by a House of Commons committee, aims to create a competitive 'internal market' within the NHS so that patients will be referred to hospitals that offer the cheapest treatment. The problem is that clinical research inevitably involves increased treatment costs. Sir Walter Bodmer, director of research for the Imperial Cancer Research Fund (ICRF), fears that, without additional government money to support these 'service costs', hospitals may be forced to cut back their clinical research programmes.

The government has accepted that some additional support for research is needed, but Bodmer describes their proposals to achieve this as "totally inadequate". The Department of Health intends to increase the money distributed to hospitals for teaching-related costs, the Service Increment for Teaching (SIFT), by about two per cent in real terms, to cover research service costs. But this money is allocated to hospitals on the basis of undergraduate medical and dental student numbers, which bear little relation to clinical research activity.

The idea to extend SIFT comes from a steering group chaired by Sir Christopher France, permanent secretary at the Department of Health. Opponents point out that the group's remit was extended from planning undergraduate teaching to considering research shortly after the government's plans for the NHS were outlined, but the research community was

never asked to take part.

ICRF proposes that instead of an increased SIFT, a new Service Increment for Research (SIFOR) should be targeted specifically to benefit those hospitals running clinical research programmes.

ICRF's proposals are supported by the Cancer Research Campaign. These two charities already divert about a third of their budgets for many hospitals into basic patient care, much more than most medical charities, and fear that the NHS bill will exacerbate the situation.

But other organizations believe that ICRF's proposals are not immediately practical. Diana Garnham, of the Association of Medical Research Charities (AMRC), explains that the data to implement SIFOR schemes are not yet available. Clinical research in the NHS is funded from a wide variety of sources and there has been no attempt to document the total level of research activity in different hospitals. AMRC would like to see something along the lines of SIFOR as a long-term goal, but a more realistic short-term approach is to suggest how the Department of Health could get an indication of where clinical research is centred, so that the distribution of SIFT could be modified accordingly.

The Medical Research Council (MRC) holds similar views. Present arrangements for the distribution of SIFT will be reviewed in 1992, and the MRC accepts that no major changes are likely before then.

Opposition to the NHS bill is also coming from the universities, which run hospital medical and dental schools. The Committee of Vice-Chancellors and Principals (CVCP) is unhappy with the proposed level of university representation in the local management of the NHS.

Peter Aldhous

SPACE RESEARCH

Japan's rocketing ambitions

Tokyo

WITH the successful launch of its lunar probe (see below), Japan's academic space agency ISAS is considering its future plans and, in particular, whether there is a need for increased cooperation with foreign agencies or its domestic big brother, the National Space Development Agency (NASDA), to aim at more ambitious missions.

In the immediate future, the situation is unlikely to change with scientific missions beyond the Earth still the prerogative of ISAS while NASDA concentrates its efforts on the launch of Earth-orbiting applications satellites. But later on, the two agencies may have to join forces.

ISAS's present plans are for the launch of a radiotelescope, using the new M-V rocket, into a 20,000-km eccentric Earth orbit in 1994 or 1995 to form a very-long baseline interferometry network with radiotelescopes on Earth. And in the next few months Jun Nishimura, director general of ISAS, says the institute will choose one of the three other missions being considered for 1996: a lunar spacecraft that will drop penetrometers containing seismographs to monitor moonquakes, release of a balloon into the atmosphere of Venus, and a mission to collect dust from the comet Wirtanen.

But the M-V rocket will only be able to send small scientific payloads to the planets and Minoru Oda, former director general of ISAS, sees it partly as carrying out "scouting missions" that could be followed by bigger projects involving international collaboration or joint projects with NASDA using its much more powerful H-2 rocket. But both Oda and Nishimura are anxious to retain ISAS's independent capability to carry out small scientific missions at low cost.

ISAS has several joint missions with the United States coming up in the next few years (see below) and collaboration might next be extended to the Soviet Union. Nishimura says that in December he had very preliminary discussions with Albert Galeev, director of the Space Research Institute in Moscow, about joining the Soviet Union and other nations in a mission to Mars in 1994 or later.

Tomifumi Godai, executive director of NASDA, agrees that ISAS and NASDA may cooperate in the launch of large scientific payloads. But NASDA has its own plans for sending remote-sensing satellites to the Moon or planets and is even considering a manned lunar base.

For at least the next decade NASDA will be fully occupied with Earth-orbiting missions, the largest being the US space station. The Japanese Experiment Module (JEM) for the station now scheduled for launch in early 1998 will cost about

¥300,000 million (\$2000 million) to develop and will have annual operating costs of about ¥40,000 million, according to Tada-aki Mochida of NASDA who presented a cost analysis for JEM at a workshop last month in Tokyo.

NASDA and STA have long hoped to separate the budget for JEM from NASDA's general budget so that the space station does not become a millstone around NASDA's neck. But the Ministry of Finance has resisted such requests, says Yasushi Horikawa, operations manager for NASDA's space station office. Unless there is a dramatic increase in NASDA's budget (which is unlikely) JEM will swallow about a third of NASDA's annual budget.

NASDA is hoping to recover some of JEM's costs by allowing Japanese private

companies to carry out experiments in JEM. But the re-phasing of the space station by the United States last year has caused a loss of confidence and Japanese companies will probably wait and see what happens before committing themselves.

NASDA sees the station as a way of developing an independent capability for manned space flight. Oda and Nishimura, on the other hand, are less enthusiastic about manned missions. Oda's personal opinion has long been that there should be greater use of robots and automation on the space station — something which may well come about because of the US decision last year to reduce the number of crew members from 8 to 4 during the first few years of operation. But if Japan is going to send men into space, "we should use them" Oda says. Nishimura says that ISAS will probably carry out some projects on the space station. **David Swinbanks**

Moon next stop

Uchinoura

JAPAN last week became the third nation to launch a spacecraft to the Moon. But besides the giant lunar programmes of the Soviet Union and the United States, Japan's efforts cost next to nothing.

Japan's moonshot is the work of the Institute of Space and Astronautical Science (ISAS), a small academic space agency affiliated with the Ministry of Education, Science and Culture. On Wednesday 24 January, one of the institute's M-3SII solid fuel rockets successfully carried a tiny 182-kg satellite into an elliptical orbit around the Earth in a night launch from the ISAS space centre at Uchinoura in southern Kyushu.

The satellite, named Hiten after a Buddhist angel, will orbit the Earth four-and-a-half times before being pulled by gravity towards the approaching Moon on 18 March. As the satellite swings by the Moon at an altitude of about 18,000 km, it will release a mini-satellite about the size of a basketball into lunar orbit.

The mini lunar orbiter will transmit information back to Earth on its temperature and position for 30 days. Meanwhile the mother satellite, after orbiting the Earth another three-and-a-half-times, will swing by the Moon again at an altitude of about 35,000 km on 10 July.

Hiroki Matsuo of ISAS who is in charge of the mission says the main purpose is not to reach the Moon but rather to test the swing-by technique that will be used in future lunar and planetary missions, such as the GEOTAIL mission, a joint project with the United States scheduled for 1992 (see 'Working with NASA'). The only scientific instrument aboard Hiten is a Munich Dust Counter which will determine the speed and size of particles that hit the satellite as it ventures up to distances of 700,000 km from Earth.

The total cost of Japan's first moonshot including the M-3SII rocket is about \$50 million dollars. **David Swinbanks**

Working with NASA

Tokyo

JAPAN and the United States have signed an agreement to launch a Japanese spacecraft into the tail of the Earth's magnetic field. The GEOTAIL mission is another example of the growing collaboration between the two countries in space.

The GEOTAIL satellite will be built by Japan's ISAS space agency and is scheduled to be launched in July 1992 by the US National Aeronautics and Space Administration (NASA) on a US Delta II rocket.

The spacecraft will examine the Earth's magnetic field on the nightside of the planet where it is drawn out into a long tail by the solar wind. The aim of the mission is to clarify energy transport and conversion in the magnetosphere, in particular the interactions between the solar wind and the magnetic field.

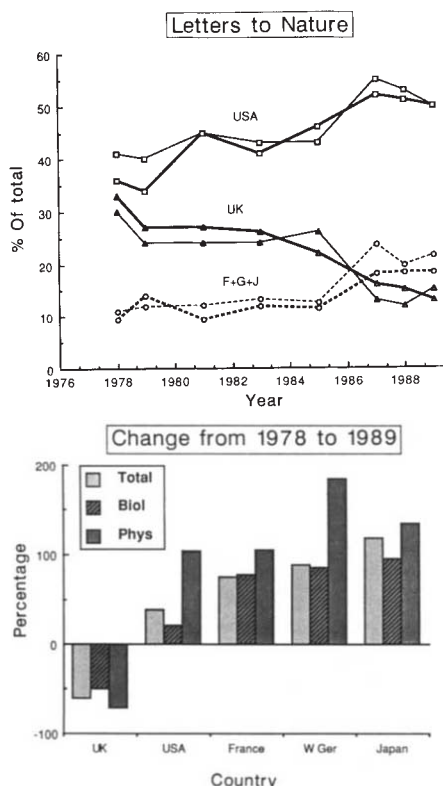
The satellite will perform a double swingby of the Moon, taking measurements in a region extending from 8 to 220 times the Earth's radius before moving into an elliptical orbit closer to the planet, in the equatorial magnetosphere.

GEOTAIL is one of several joint US-Japanese space projects proposed for the next few years. At the end of last month, the Japanese cabinet approved an initial budget for a project to monitor tropical rainfall with a US satellite to be launched on a Japanese H-2 rocket in the mid-1990s. Next year, ISAS will launch an X-ray satellite, SOLAR A, to monitor solar flares with US and Japanese X-ray telescopes. In 1995, Japan's other space organization, the National Space Development Agency (NASDA), will launch its Advanced Earth Observation Satellite (ADEOS) which will monitor the Earth's climate and ozone layer using sensors developed by NASA, NASDA and the European Space Agency. And, of course, Japan is a major participant in the US space station project. **David Swinbanks**

Index of decline of British science

SIR—*Nature* has an 'impact factor' almost ten times greater than that of other journals; so those working at the leading edge of science try to publish in it. As *Nature* has offices in Tokyo, Munich, Washington and London, it should provide a good sample of world science. This letter is an analysis of Letters published in *Nature* since 1978 to see if trends could be identified; for these might be an early indication of the future health of science in the countries studied.

Results were analysed separately for the physical and biological sciences. The figure shows that the percentage of letters in each section originating in the United Kingdom in 1989 has fallen to about half that in 1978, whereas those from the United States, France, Germany and Japan have increased. The number of



The upper part shows the percentage of all (dark lines) or 'biological' letters originating in the indicated countries. Those from France, Germany and Japan, all rising, have been added. The lower part shows the percentage change for each country from 1978 to 1989, as total, biological or physical. 'Letters to Nature' in the first five issues of eight of the years from 1978 to 1989 were categorized into physical or biological sciences, and their country of origin apportioned according to the addresses given by the authors. Because of the small numbers involved, only those from the United Kingdom, the United States, France, West Germany and Japan were counted separately. The total sample size for each year was around 100, making up about 10 per cent of each year's total.

letters from the United Kingdom and the United States is now roughly proportional to their populations. The large rise in those from Japan does not support the view that the Japanese are uninterested in basic science. The type of biology letter published seems to have changed with time: the early ones described results obtained with simple equipment, whereas many of the later ones describe the sequences of DNA and other molecules, results requiring large machines and an infrastructure to run them. The abrupt drop in the biology letters from the United Kingdom starting around 1985 may reflect this change in the expense of modern biology and the inadequacy of our current level of funding.

A detailed examination of the subset of results published in high quality journals such as *Nature* might provide better evidence for the present state of science in various countries than the blanket approach currently used.

J. F. LAMB

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Problems of South Africa

SIR—It is unfortunate that the Commentary by File *et al.* on South African education (*Nature* 341, 96; 1989) was immediately followed by a letter from Mr John Ormerod (341, 99; 1989) supporting an academic boycott of South African science. The crying need there is for some sort of 'constructive engagement' that seeks development along internationally acceptable lines. The black leaders that Ormerod hears in Oslo may see political power as paramount, but the heart of the *toy-toy* (demonstration) knows that political gain will count for little without equality of educational and economic opportunities. As your article shows, it is still the universities designated as black that contribute most to correcting this imbalance in the tertiary sector, yet it is precisely these institutions that will suffer most from the effects of such a boycott, as the liberal white universities, already excellent self-publicists, will readily find ways to avoid them, while the Afrikaners simply do not care.

My fear regarding "People's Education", with its emphasis on community relevance, is that it will deny black scientists any participation in science sufficiently fundamental or challenging to let them feel a part of a wider scientific community. The result would in effect be a

self-imposed racial stereotype, but it would be accepted without qualms by white South Africans, as the liberals would see it as giving the people what they want, the *verkramp* (conservative) as giving blacks what they deserve. Unless outsiders like myself prevent the isolation of South African science by boycott, this damaging insularity will have become eradicable by the post-apartheid era. If anyone really wishes to "stick his neck out" on the role of scientists in this particular conflict, I can readily find him a place beside me on the front line.

JOHN S. RUTHERFORD

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SIR—There are several inaccuracies in Michael Cherry's article on the South African research budget (*Nature* 342, 605; 1989).

The national laboratories of the Council for Scientific and Industrial Research (CSIR) were never taken away from the Foundation for Research Development (FRD), because the FRD has never maintained its own laboratories; it is a funding body and not a research organization. There has not been a shift of emphasis from basic to applied research within the FRD; in fact the lion's share of funding still goes to the core programme which mostly supports basic research. Minister Gerrit Viljoen has not gone on record in favour of either basic or applied research. The National Accelerator Centre's budget is nowhere near 34 per cent of the CSIR's budget. It is, in fact, 6.7 per cent of the total budget.

C. F. GARBERS

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■ The errors of which Dr Garbers complains were introduced in the sub-editing of this story for publication. — Editor, *Nature*.

The coming ice age

SIR—The study of the oxygen-18 content of the CaCO_3 shells of marine organisms by, for example, Emiliani (*Science* 202, 627; 1978) has shown convincingly that we are living at the zenith of the present Interglacial. The presence of an atmospheric CO_2 blanket should therefore be considered as a blessing for the coming 50,000 years.

A. J. RUTGERS

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The peripatetic fossils: part 5

John A. Talent

Replying to articles published in last week's *Nature*, Talent re-asserts that V. J. Gupta is responsible for corrupting the palaeontological literature on the Himalayas.

V. J. GUPTA'S and J. B. Waterhouse's responses^{1,2} to my Commentary article³ addressed none of the fundamental issues raised either there or in an earlier publication⁴. Gupta⁵ has also failed to account for the disturbing evidence presented by three of his co-authors⁶⁻⁸. Briefly, the most serious charges sidestepped are reporting discoveries that cannot be confirmed by subsequent workers; reporting the same specimens from more than one locality ('recycling' them) in support of new and sometimes startling stratigraphic alignments; and giving extremely vague and often misleading 'data' for localities.

My colleagues and I have stressed⁴ that we undertook our examination of the palaeontological and geological literature on the Himalayas "without regard to personalities but purely in order to set the record straight so that the next generation" might be able to confront the Himalayan database afresh. In the previous articles^{3,4}, we deliberately underplayed the situation, giving only a sample of the disinformation known to us at that time. 'Recycling' of specimens is more pervasive than we had realized earlier⁹. That the edifice of disinformation has greater dimensions and perhaps more serious implications than we had imagined is brought out by three of Gupta's co-authors⁶⁻⁸ and in correspondence we have received.

Gupta's co-authors are understandably disturbed; some have publicly acknowledged having been duped⁶⁻¹⁰. Waterhouse, however, his principal co-author with 19 joint publications, is reluctant to follow their lead. In his article², Waterhouse plays down the damage done by Gupta's 25-year enterprise — at last count 4,147 pages of print in 405 publications, 144 of which were written by him alone, the rest with 117 co-authors (56 of whom are non-Indian).

In stressing the dearth of new species, Waterhouse argues that the significance of Gupta's *oeuvre* is thereby diminished. Ironically, the data presented by Waterhouse are consistent with a very different interpretation. The problem is not one of overspeciation, the sort of minor aberration that does little permanent damage to the palaeontological database. Rather, it is the host of suspect reports, many of which are based on the sort of materials often found in teaching laboratories, that are readily obtained from colleagues "for teaching purposes" (G. D. Webster, per-

sonal communication), that are easily collected from well-known localities, or that can be purchased for nominal sums from rock shops and curio counters around the globe^{3,4}. It is therefore understandable that Gupta's contribution as regards numbers of 'new species' is not excessive; his reports mostly concern well-known species, neatly explaining the lack of novelty.

That Gupta sent Waterhouse Cambrian and Carboniferous-Triassic collections

Figures of Middle Cambrian trilobites from Mt Stephen, British Columbia¹², cannibalized to document a 'discovery' at Zachaldor, Kashmir¹¹. The specimens are in the US National Museum.

Identification	Rasetti 1951	Gupta 1967
<i>Ogygopsis klotzi</i> (Rominger)	Pl.29 Fig.7	Fig.1
<i>O. klotzi</i> (Rominger)	Pl.29 Fig.8	Fig.2
<i>O. klotzi</i> (Rominger)	Pl.29 Fig.6	Fig.3
<i>Tonkinella stephensis</i> Kobayashi	Pl.31 Fig.18	Fig.4
<i>O. klotzi</i> (Rominger)	Pl.21 Fig.3	Fig.5
<i>Wenckhemnia sulcata</i> Rasetti	Pl.11 Fig.15	Fig.8
<i>O. klotzi</i> (Rominger)	Pl.12 Fig.5	Fig.9

from well-known localities is irrelevant; it is the anomalous ones that matter. Some of the Gupta-Waterhouse faunas are obviously Himalayan but the precise provenance of many of them needs confirmation.

Waterhouse's approach² is to discount specific accusations against Gupta by recounting innocuous experiences with Indian materials. This 'inductive' method for restoring faith is logically flawed. Waterhouse claims that Gupta's publications on the Cambrian, Carboniferous, Permian and Triassic of the Himalayas are positive contributions. The evidence already published^{3,4,9} indicates otherwise. A further selection of examples for each period highlights this. Seven of the nine illustrations in Gupta's documentation of Cambrian trilobites from Zachaldor¹¹ have been cannibalized from a monograph by Franco Rasetti on trilobites from the Rocky Mountains¹² (see table). On four occasions the same specimens of Carboniferous corals have been assigned to widely separated localities (ref. 13 Pl.36, Figs 1, 4; ref. 14 Fig. 1d,e; ref. 15 Figs 1-3; ref. 16 Pl. 2, Figs 2, 3 and 5). The sources of the Waterhouse-Gupta Bijni

and Ralaking volcanics Permian brachiopod faunas^{17,18} (collectors unspecified) are problematic (M. Gaetani, personal communication); both are phantom localities, though similar materials can be found elsewhere in the region (R. S. Chaturvedi & J. A. T., unpublished information). The source of the Tidong valley Permian faunas is also problematic, as it has been suggested that Gupta may never have been there⁸. There are numerous examples of recycling Triassic conodonts (ref. 19 Fig. 10p; ref. 20 Pl. 1, Fig. 5; ref. 21 Pl. 3, Fig. 5). Of what value then is Waterhouse's assertion² that for the above four periods "the general Himalayan source of Gupta-provided material has been verified independently" by him?

Waterhouse also insinuates that I have no knowledge of relevant collections in India. As early as 1970 I examined Cowper Reed's Ordovician-Devonian collections at the Geological Survey of India in Calcutta in order to ascertain the reliability of Reed's illustrations; and in August 1971, late January 1976 and late 1980, I examined such Gupta materials as were on display in the museum at Chandigarh. On the second occasion, a request to Gupta to examine a pivotal specimen he had reported²², *Calceola sandalina* (Linné), received such an evasive response that it was clear that access to other suspect materials was out of the question. To "work through" all museum collections in India, as Waterhouse suggests, would be a tedious and irrelevant exercise.

Waterhouse² asserts that a number of Gupta's co-authored articles "hold their validity". But which ones? In the 19 papers co-authored with Gupta, Waterhouse accepted Gupta's locality data. In view of the spurious nature of so many localities given by Gupta, what basis can there be for accepting others as valid without checking the precise spots in the field where his collections are said to have originated? How "detailed morphological studies", "comparative analysis", "counts" and "distribution studies" (ref. 2) might clarify the questions at issue escapes me, especially when precisely the same specimens have been used over and over again^{3,9}.

A number of Gupta's statements^{1,5} call for response. First, listing the names of others present on particular expeditions is irrelevant unless those named are willing to provide testimony that it was they who

collected the anomalous items or witnessed others doing so. We have not implied that any of Gupta's co-authors are responsible for the stratigraphic and locational deficiencies in the papers concerned.

Second, Gupta¹ cites A. K. Pal's reports of 'graptolites' as though they were valid; the graptolites have proved to be inorganic (D. E. B. Bates, personal communication). We assumed this was common knowledge among those involved in Himalayan geology. Contrary to Gupta's claim¹, we also took it to be widely known that the late Professor M. R. Sahni never visited the Gupta graptolite and Muth Quartzite sites in Kashmir; this has been verified by his son²³.

There is no question that occasional poorly preserved shelly fossils occur in the Muth Quartzite; this has been known for over 80 years in Spiti²⁴, but neither we⁴ nor subsequent workers²⁵ have found shelly fossils "half to two-thirds of the way up the Muth Quartzite" in the key Gugaldar area. Some — perhaps all — of Gupta's supposed Middle Devonian shelly faunas from the Muth Quartzite of Kashmir are Late Ordovician or Early Silurian materials levitating to a more interesting horizon⁴.

Third, Gupta's contributions on the Palaeozoics of the Kathmandu region^{26,27} are noteworthy for a lack of structural data from which one might infer the structural complexity he asserts¹. Our observations accord well with Stöcklin's²⁸ structural and stratigraphic analysis. Gupta's assertion that he reported a conodont fauna indicative of the *triangularis* Zone from Phulchauki finds no support in the cited reference²⁰.

Gupta⁵ is correct in his statement that fossils preserved in haematite (or goethite) occur on Phulchauki. But what he did not point out is that this is not at high altitude, like Khimokul La, and that these fossils occur as extremely friable moulds in a sedimentary iron ore deposit. They are quite unlike the hard haematite moulds generated in deep weathering profiles, such as those found at Erfoud in Morocco. Statements by Gupta⁵ and Waterhouse² should be read in conjunction with the report⁸ that neither limestones nor ammonoids, much less haematite moulds of Devonian ammonoids, can be found at Khimokul La where Gupta²⁹ claimed to have found them.

Fourth, neither the Sweet-Gupta⁴ nor Clark-Gupta correspondence (shown to me by Professor D. L. Clark) provides supporting evidence for Gupta's assertion that either of them confirmed his identifications of Triassic conodonts, supposedly from Tandi in Lahaul. Gupta's initial 'Tandi list' sent by the editor of *Journal of Geology* to C. McA. Powell and P. J. Conaghan on 10 January 1974 has no species in common with identifications reported by Sweet and Clark in

letters to Gupta, respectively 12 months and 6 months previously (J. A. T., unpublished information), though there is some convergence in the published list³⁰.

Fifth, contrary to Gupta's assertion¹, my colleagues and I did respond to his invitation, made at the Second International Symposium on the Devonian System in Calgary on 20 August 1987, to visit Chandigarh to examine his collections. Our response was unequivocally negative; we felt that such an exercise would be pointless. As we have stated⁴, "in circumstances such as these, no significance can be attached to locality information or any other data given on museum labels".

Sixth, our inference⁴ that Gupta's report²⁹ of early Late Devonian conodonts with latest Devonian ammonoids some 15 million years younger at Khimokul La was unacceptable is now confirmed by field observations⁸. Seventh, the plea of having to "depend on old and inaccurate maps" lacks plausibility and has been addressed elsewhere^{3,4}.

Finally, I turn to Gupta's response⁵ to three co-authors turned critics⁶⁻⁸ (here I pass over both Janvier's comments in the same issue³¹, and Gupta's reply, because Janvier was largely concerned with broader aspects of the debate).

In replying to A. D. Ahluwalia, Gupta argues that illustrations of Carboniferous corals in two papers, published a mere two months apart, were somehow swapped⁵. There is no evidence of incongruities between text and illustrations in these papers; even if there were, his excuse provides no explanation for using the same two specimens on four occasions, on each occasion giving a different 'locality'¹³⁻¹⁶.

In responding to S. B. Bhatia, Gupta insists he "had no interest in ostracodes or microfossils in 1972". In the period 1967-72 he published ten papers on microfossils including two on ostracodes, both with Bhatia^{32,33}.

Gupta's assertion⁵ that in 1974 he visited Kinnaur — an area a third of the size of Wales — is evasive as regards the critical Tidong Valley-Khimokul La portion of Kinnaur. In 1982 it was implied¹⁷ that he, Waterhouse or both of them had made "fresh collections" in that area, subsequent to a paper published two years previously³⁴. Bassi's testimony⁸ — absence of entries in registers at security checkpoints and erroneous stratigraphy — is evidence that until at least 1984 Gupta had not visited this particular area.

An expedition of Indian scientists to the localities in question might be desirable³⁵. But from the evidence that has already been brought forward⁶⁻⁸, which is in line with what I and my colleagues have presented elsewhere^{3,4,9}, it seems that such an expedition would be a formality. If anything else needs to be done it should

involve isotope investigations. Specimens reported from each locality in the Himalayas will have trace-element or isotopic signatures different from specimens of the same species from elsewhere in the world. The specimens whose provenance is under debate^{3,4,6-9} should be loaned, through a neutral body such as the Society for Scientific Values, for comparisons to be made by an independent laboratory.

All field books, laboratory registers and specimens, including relevant thin sections of corals and micro-slides of conodonts, should be made available to examining commissions so that all of the questionable field data can be checked. Especially interesting would be a tabulation of materials that have been recycled or are illustrated by pictures from other people's monographs.

My conclusion remains as before — that all contributions authored or co-authored by Gupta should be ignored, and syntheses that have taken his 'data' to be reliable should be used with extreme caution. □

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- Gupta, V.J. *Nature* **342**, 11-12 (1989).
- Waterhouse, J.B. *Nature* **343**, 305-307 (1990).
- Talent, J.A. *Nature* **338**, 613-615 (1989).
- Talent, J.A., Goel, R.K., Jain, A.K. & Pickett, J.W. *Cour. Forsch.-Inst. Senckenberg* **106**, 1-57 (1988).
- Gupta, V.J. *Nature* **343**, 307-308 (1990).
- Ahluwalia, A.D. *Nature* **341**, 13-15 (1989).
- Bhatia, S.B. *Nature* **341**, 15 (1989).
- Bassi, U.K. *Nature* **341**, 15-16 (1989).
- Talent, J.A., Brock, G.A., Engelbreiten, M.J., Kato, M., Morante, R. & Talent, R.C. *Geol. Soc. India J.* **34**, 575-586 (1989).
- Lewin, R. *Science* **244**, 277-279 (1989).
- Gupta, V.J. *Punjab Univ. Res. Bull.* **18**, 275-277 (1967).
- Rasetti, F. *Smithsonian Misc. Publ.* **116**, 1-277 (1951).
- Gupta, V.J. *Punjab Univ. Cent. Adv. Stud. Geol. Pub.* **9**, 1-100 (1971).
- Gupta, V.J. *Indian Geol. Ass. Bull.* **18**, 137-139 (1985).
- Gupta, V.J. *Geol. Soc. India J.* **27**, 223-224 (1986).
- Kato, M. & Gupta, V.J. *J. Fac. Sci. Hokkaido Univ. IV* **22**, 399-424 (1989).
- Waterhouse, J.B. & Gupta, V.J. *Recent Res. Geol.* **4**, 410-437 (1978).
- Gupta, V.J. & Waterhouse, J.B. *Recent Res. Geol.* **5**, 31-49 (1978).
- Budurov, K.J. & Gupta, V.J. *Indian Geol. Ass. Bull.* **21**, 21-39 (1988).
- Kachroo, R.K., Gupta, V.J. & Budurov, K.J. *Recent Res. Geol.* **8**, 271-273 (1982).
- Gupta, V.J. *Indian Geol. Ass. Bull.* **16**, 51-62 (1983).
- Gupta, V.J. *Punjab Univ. Res. Bull.* **21**, 1-22 (1970).
- Sahni, A. *Nature* **342**, 338 (1989).
- Hayden, H.H. *Geol. Surv. India Mem.* **36**, 1-129 (1904).
- Srikantia, S.V. & Bhargava, O.N. *Geol. Soc. India J.* **24**, 363-377 (1983).
- Gupta, V.J. *Himalayan Geology* **5**, 152-168 (Wadia Inst. Himalayan Geol., Delhi, 1975).
- Gupta, V.J. & Chhetri, V.S. *Chayanica Geologica* **3**, 133-146 (1977).
- Stöcklin, J. *J. geol. Soc. Lond.* **137**, 1-34 (1980).
- Gupta, V.J. & Erben, H.K. *Paläont. Zeitschr.* **57**, 93-102 (1983).
- Gupta, V.J. *J. Geol.* **83**, 127-128 (1975).
- Janvier, P. *Nature* **341**, 16 (1989).
- Jain, S.P., Gupta, V.J. & Bhatia, S.B. *Indian Geol. Ass. Bull.* **2**, 33-35 (1969).
- Jain, S.P., Bhatia, S.B. & Gupta, V.J. *Himalayan Geol.* **2**, 168-187 (Hindustan, Delhi, 1972).
- Chopra, S., Gupta, V.J., Bassi, U.K. & Ahluwalia, A.D. *Punjab Univ. Cent. Adv. Stud. Geol. Pub.* **12**, 337-339 (1980).
- Radhakrishna, B.P. *Geol. Soc. India J.* **34**, 561-563 (1989).

Two gales do not make a greenhouse

Northern Europe, recovering from last week's gales, is ready to believe in a global warming caused by an excess greenhouse effect. But that is not the reason which an international convention must be hurried along.

MANY of the traditionally white clapboard churches of New England are distinguished by their curiously truncated spires, mere stumpy bases which are spires only in the mind's eye. In the locality, the explanation is well-known. For most of the nineteenth century and for much of this, New England was free from Caribbean hurricanes. But then, in 1938, the hurricane track changed, and violent storms that year truncated many pre-existing church spires. Cautious congregations decided to leave things more or less as they were left by the winds, but the more dutiful, who rebuilt, have not been seriously incommoded. Since the 1960s, hurricanes on the East Coast of the United States have followed yet a different track.

That is one comment on the meteorology of northern Europe during the past few weeks. Last Thursday, in particular, winds associated with a deep but narrow depression moving east up the English Channel caused havoc that killed close on fifty people in southwest Britain, The Netherlands and northern West Germany. By the end of the week, the sequence of depressions lining up in the North Atlantic had created a sense of excited caution among Europe's weather forecasters. Similarities with the great wind of October 1987, perhaps technically a hurricane, were too close for comfort.

What has happened to change the usual pattern of winter weather in Northern Europe, when anticyclones can usually be relied upon to anchor themselves over Scandinavia, bringing freezing weather and occasional blizzards? Especially because ski resorts in the Alps have been moaning for months (as they were last year) that there is not enough snow on the lower slopes, it is natural to look for some all-embracing explanation. What, in present circumstances, can it be?

Mrs Margaret Thatcher, the British prime minister, has several faults, impulsiveness the most endearing of them. Last weekend she put into words a thought that has been in knowledgeable people's minds for some time, that the unseasonable weather and now the wind is a sign that the greenhouse effect, once a hypothesis, has become reality. "It is strange to have two hurricanes in this manner", she told an audience at Wigan, in northwest England. "We've also had two mild winters. We don't know whether it's the greenhouse effect yet... but it's a double reminder that

we must take every action to keep the outer atmosphere intact."

What Mrs Thatcher said, of course, is strictly proper and entirely correct. It is strange, in the sense of being unfamiliar in northern Europe, that there has been so much high wind, and that two consecutive winters have been exceptionally mild. It was proper of her quickly to imply that these isolated pieces of information do not constitute a proof that the greenhouse effect is already with us, although purists may complain that the word "yet" begs the question. And it is natural that a politician laudably committed to the notion that something drastic will have to be done to fend off the reality of the greenhouse effect should have seized on people's sense of alarm to bend their inclinations to her purpose.

The danger, for prime ministers no less than for others with the environmental bit more professionally between their teeth, is that even indirect and properly qualified assertions that transitory events are evidence for more durable physical phenomena may backfire. Will last weekend's audience put the greenhouse effect at the back of its mind if there should be no near-hurricane in the next winter season, or if dedicated skiers are able to take their exercise without travelling very far?

That hurricanes, or near-hurricanes, may be signs of the reality of an excess greenhouse effect is plausible enough. If it were the case that depressions over the North Atlantic are formed off the eastern coast of North America as a consequence of the contrast of temperature between the land and the sea, and if they are then intensified as a result of the accumulation of thermal energy in the ocean, the events of the past few weeks or even years would be explained (in European eyes) if global warming were already a reality. One might even plead Le Chatellier's principle to argue that, if infrared radiation from the surface of the Earth is impeded by greenhouse gases, the atmospheric system will use other mechanisms for lifting heat in the atmosphere (which depressions accomplish by depositing latent heat at the level of the clouds from which their precipitation is released).

But the harsh truth is that even this season's string of severe depressions reaching northern Europe is within, if near the extreme of, the recorded pattern of European weather, while there is no evidence that the sea-surface temperatures

in the North Atlantic have markedly increased in the past few years. The truth is that the evidence to which Mrs Thatcher drew attention is consistent with, but is not a proof that, there is already an excess greenhouse effect in being. It is equally important that markedly different weather next winter will not be a sign that the threat has gone away.

How, in these circumstances, to create the climate of public opinion that will provide a fair wind for the international conventions to regulate all greenhouse gases for which there is an urgent need? The cumbersome machinery for reaching international agreement is, fortunately, in place. This week's meeting of an international technical panel in Washington (see page 401) is one of several planned in advance of the negotiating session due to be held at Geneva in November.

But it will be a great surprise, and might even be mistaken, if that meeting should arrive at more than a skeleton of a convention. It will be a surprise because the serious political issues that must eventually be tackled — the distribution of carbon dioxide quotas between different countries, for example — have hardly as yet been identified. An agreement would be unwise because — the uncertainties in the predictions of global warming apart — there remain huge uncertainties about the causes of the accumulation of some of the greenhouse gases, methane, for example, and about the best strategy for abating them.

The importance of tropospheric chlorofluorocarbons (CFCs) as greenhouse gases (as distinct from thieves of stratospheric ozone) is well-known, but the economic cost of regulating them is a much less well-known quantity. The 1987 Montreal Protocol to the Vienna Convention on the Safeguarding of the Ozone Layer requires a standstill in production of CFCs, but if there are much less long-lived substitutes in the offing, a complete ban on long-lived chemicals would be more prudent, and cheaper.

That shows that those who draft the next convention will have to create a framework for binding agreement between sovereign states whose details can be changed as knowledge increases and technology advances. That must be a formidable task. Among international conventions, it will be uniquely difficult, which is the chief reason for hurrying.

John Maddox

Cytoskeleton on the move

Peter J. Hollenbeck

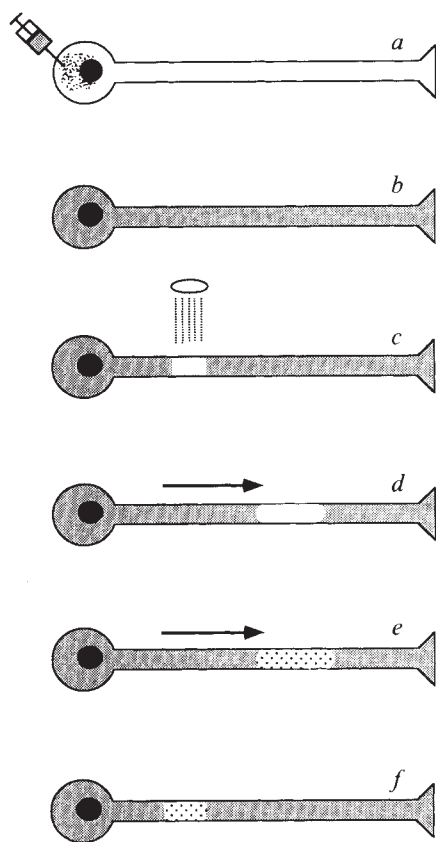
NEURONS, like other eukaryotic cells, must strike a balance between structural stability and plasticity. One agent of this compromise is the intracellular system of polymeric protein filaments called the cytoskeleton. In the neuron, these filaments include microtubules, which are composed of tubulin subunits; microfilaments, composed of actin; and neurofilaments, which contain three different protein subunits. By controlling the assembly, stability and interactions of these filaments, neurons use the cytoskeleton to maintain their shape and position as well as to change them — to shuttle their internal contents about us well as to exclude them from certain regions. On page 479 of this issue¹, Okabe and Hirokawa report the use of a powerful labelling technique to observe the behaviour of cytoskeleton proteins in living neurons. Their results show that two of the cytoskeletal polymers — microtubules and microfilaments — do not translocate in the axon, but do undergo assembly and disassembly there.

This result is of interest because of the special architectural challenge faced by neurons, in that they elaborate axonal processes which are not only highly elongated and subject to extreme mechanical stress, but which are also segregated from the synthetic machinery of their parent perikaryon, the region containing the nucleus, endoplasmic reticulum and Golgi apparatus. As might be expected, the axonal cytoskeleton has features that are viewed as adaptations to this situation: its polymers are densely packed and unusually stable, and their constituent proteins are synthesized in the perikaryon and then conveyed distally along the length of the axon. What form the moving cytoskeletal proteins take in the axon, where they are assembled, and how they provide the axon with its particular balance of stability and plasticity are profound but difficult questions.

We owe most of our knowledge of the axonal transport of cytoskeletal proteins to a large body of pulse-labelling studies performed both in mature nerves and in cell culture. In these studies, neuronal perikarya were exposed to radiolabelled protein precursors, and the transport rate, solubility properties and modifications of labelled proteins were analysed over time².

From this approach, we know that the constituent proteins of the cytoskeleton show reduced solubility soon after entering the axon³, and that they are transported slowly (at rates of millimetres per day) along the axon towards the nerve terminal at two different velocities⁴.

Several models of the neuronal cytoskeleton have arisen from this research, the models differing in where it is proposed that the cytoskeletal filaments are assembled and what form the moving protein takes. In one view, cytoskeletal proteins are assembled into polymers soon after entering the axon; the transported protein is thus polymeric, either in a highly stable crosslinked matrix^{4,5}, or in the form of individual sliding polymers which undergo some exchange with a pool



Schematic representation of the molecular cytochemical technique used in refs 1 and 12. *a*, Fluorescently labelled subunits of cytoskeletal polymers are introduced into neurons by injection into the perikaryon. *b*, After an interval, the subunits are incorporated into polymers throughout the axon. *c*, A spot is bleached on the fluorescent cytoskeletal array using a laser; the fate of the spot can be followed over a period of hours. Three possible results are shown. *d*, If cytoskeletal polymers are translocating distally along the axon, then the spot should move, spreading as it goes due to variation in transport rates of individual filaments. *e*, If polymers are both translocating and exchanging their bleached subunits for fluorescent ones that are free in the cytoplasm, the spot will both translocate and recover its fluorescence. *f*, If they are exchanging subunits but not translocating, the spot will recover its fluorescence without moving.

of soluble subunits^{6,7}. Another proposal⁸ is that a large part of the cytoskeleton is stationary, and places less constraint upon the form of the moving protein, which could consist of subunits, polymers or insoluble non-polymeric structures⁹.

These hypotheses can now be tested directly using a variant of the technology of molecular cytochemistry¹⁰. In this procedure, subunits of cytoskeletal proteins are purified, coupled to a fluorescent dye molecule and introduced into living neurons where they become incorporated into polymers. The result is a labelled cytoskeleton which can be observed, *in vivo*, by fluorescence microscopy. An additional twist involves allowing the fluorescently tagged subunit proteins to become incorporated into polymers uniformly throughout the axon, and then bleaching a narrow zone on the axonal array with a powerful light source¹¹. The subsequent behaviour of this zone yields information about both the movement of polymers and their assembly and disassembly: if cytoskeletal polymers are translocating along the axon, movement of the bleached zone should be evident; if polymers are in equilibrium with a pool of subunits, then the zone should recover its fluorescence as labelled subunits are incorporated into polymers in the bleached region (see figure).

In the study reported in this issue¹, Okabe and Hirokawa carried out this analysis for microtubules and microfilaments in the axonal cytoskeleton of sensory neurons. They injected fluorescently labelled tubulin or actin into the perikarya of these cells, and found that, after a few hours, the subunits were incorporated into polymer throughout the axons. When the authors bleached a zone on the microtubule array and followed its fate, they found that it failed to translocate or spread along the axon. This is consistent with the results obtained by Lim *et al.* using neuron-like cell lines¹², and shows that microtubules are not undergoing movement — or that if they are, it is almost two orders of magnitude slower than the rates determined by pulse labelling for the tubulin component of axonal transport.

Okabe and Hirokawa quantified the recovery of fluorescence of the bleached microtubule zone and found a time course of recovery that suggests a two-component process. The first stage is rapid but partial, and is ascribed to the diffusion of fluorescent tubulin subunits into the bleached region. The rest of the recovery is much slower, and the authors suggest that it represents the exchange of fluorescent subunits into the bleached region of microtubules. This interpretation is supported by the data of Lim *et al.*¹², who found that the recovery of fluorescence of a bleached microtubule zone was faster in regions of the neuron

COSMIC STRUCTURE

where microtubules are known to be more labile. Okabe and Hirokawa then repeated the labelling and bleaching procedure with microfilaments, and obtained similar results — a bleached zone on the microfilament array recovers without perceptible translocation or spreading, and with a two-component time course that is much faster than that of microtubules and that is again taken to represent subunit diffusion followed by exchange into polymer.

All well and good. But if microtubules and microfilaments are stationary, which structures are responsible for the slow flow of their constituent proteins outwards along the axon? Okabe and Hirokawa suggest that slow axonal transport can be explained by the movement of soluble subunits or oligomers that are in equilibrium with their respective polymers. As with molecules subjected to chromatography, the tubulin or actin subunits' average rate of movement along the axon would be determined by how much time they spend as part of the stationary phase (microtubules or microfilaments), which would depend on the rate of exchange between free subunits and polymers. The differences in transport rate of actin and tubulin would thus be reflections of the different rates of subunit-polymer exchange measured by bleaching and recovery.

For some readers, this may bring to mind the unitary hypothesis of axonal transport put forward by Ochs¹³, in which proteins achieve different transport rates by having different affinities for a single moving vector. If so, they will no doubt wonder whether the techniques of molecular cytochemistry will reveal a relationship between rates of subunit exchange and transport when applied to other components of the axonal cytoskeleton, such as the neurofilaments or the proteins that are co-transported with actin. □

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Texture may seed galaxy growth

John L. Friedman and Michael S. Morris

THE astonishing uniformity of the cosmic background radiation and the corresponding uniform expansion seen in the redshifts of distant galaxies are a striking confirmation of the simplest cosmological model of general relativity, a symmetric, homogeneous, expanding Universe. But this very uniformity is difficult to reconcile with the obvious clumping of matter into galaxies and clusters of galaxies. Neil Turok, of Princeton University, proposes an exotic new mechanism by which defects in the very early Universe could account for this discrepancy¹.

The background radiation (measured today, roughly 10^{10} years after the Big Bang, as a black-body spectrum of temperature around 3 K) is made up of light which last touched matter only 100,000 years after the Big Bang, when it and the whole Universe were at a temperature of a few thousand degrees kelvin. At angular separations greater than one arcminute, this radiation is observed to be isotropic to one part in 10^4 . That means the Universe itself 100,000 years after the Big Bang could not have been more clumpy (could not have had over- or under-densities greater) than about one part in 10^4 on scales of a hundred megaparsecs or more. If one tries to build an evolutionary model for the Universe using only the kinds of matter we know, primarily baryons, neutrinos, electrons, light and gravitational waves, there does not yet seem to have been enough time to form galaxies, starting from such small density fluctuations. The problem is particularly severe if the recently discovered large-redshift quasars (see Carswell and Hewett's recent article²) are nuclei of typical galaxies³.

This fundamental difficulty has forced cosmologists to entertain various exotic alternatives for how to seed and subsequently grow galaxies. Some of the more popular involve cosmic strings, matter at extremely high density trapped in long string-like loops. These loops oscillate at

speeds near that of light, and gradually shrink as they radiate away their energy. The string-like structure persists until the loop has shrunk to a radius as small as its width, and in that time the excess density can have a strong enough gravitational field to seed a galaxy or a cluster of galaxies^{4,5}. The new suggestion by Turok, one of the most active proponents of the string approach to galaxy formation, is that a related structure, called texture, might similarly provide high-density seeds for galaxies and clusters¹.

Strings and texture are two examples of topological defects, patterns frozen into the fields that thread space. What we call a vacuum is not devoid of structure: the symmetry of the four forces — gravitational, electromagnetic, weak and strong — is presumed to have been successively

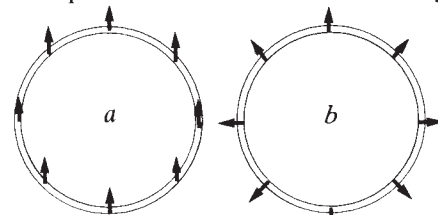


FIG. 2 *a*, Arrows on the left circle indicate a configuration of directions for the Higgs field with winding number zero. *b*, Arrows on the right circle indicate a Higgs field configuration of winding number one. The two configurations are not continuously deformable into one another (see Fig. 3).

broken in the early history of the Universe by a sequence of phase transitions as the Universe expanded and cooled. A similar breaking of symmetry is familiar from the cooling of ferromagnets. In iron, for example, at high temperatures (above 1,043 K), the spins on neighbouring atoms are randomly orientated, because their kinetic energy is more than enough to jar their spins out of alignment. When the temperature drops below 1,043 K, however, the kinetic energies become too small to disrupt the alignment, and neighbouring iron atoms pick out one direction in which to point. Cooling is typically too fast to allow the most distant atoms time to align, and one is left with local domains, each frozen into its own direction of magnetization (Fig. 1).

The fields that are thought to thread the cosmological vacuum and break the symmetry of the four forces are called Higgs fields (or Nambu-Goldstone fields). They, like the spin of the atoms in a ferromagnet, can be thought of as arrows at each point in space (although the arrows need not have three components). It is fairly easy to understand topological defects like texture if one thinks of a model universe that has fewer dimensions

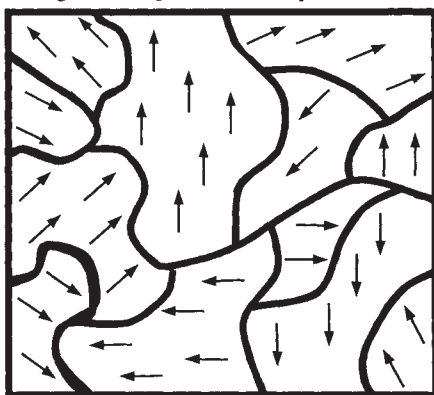


FIG. 1 Domains of uniform magnetization (spin alignment) in a cooling ferromagnet.

- Okabe, S. & Hirokawa, N. *Nature* **343**, 479–482 (1990).
- Lasek, R.J. & Brady, S.T. *Meth. Cell Biol.* **25**, 365–398 (1982).
- Black, M.M., Keyser, P. & Sobel, E. *J. Neurosci.* **6**, 1004–1012 (1986).
- Black, M.M. & Lasek, R.J. *J. Cell Biol.* **86**, 616–623 (1980).
- Lasek, R.J., Garner, J.A. & Brady, S.T. *J. Cell Biol.* **99**, 2125–221s (1984).
- Willard, M. & Simon, C. *Cell* **35**, 551–559 (1983).
- Lasek, R.J. *J. Cell Sci. suppl.* **5**, 161–179 (1986).
- Nixon, R.A. & Logvinenko, K.B. *J. Cell Biol.* **102**, 647–659 (1986).
- Weisenberg, R.C. *et al. Science* **238**, 1119–1122 (1987).
- Taylor, D.L. & Wang, Y.-L. *Proc. natn. Acad. Sci. U.S.A.* **75**, 857–861 (1978).
- Keith, C.H. *Science* **235**, 337–339 (1987).
- Lim, S.S., Sammak, P.J. & Borisy, G.G. *J. Cell Biol.* **109**, 253–263 (1989).
- Ochs, S. in *Recent Advances in Myology* (eds Bradley, W.G. *et al.*) 189–194 (Excerpta Medica, Amsterdam, 1975).

than ours. In two spatial dimensions, the simplest finite universe is a two-dimensional spherical surface, like a spherical balloon, while in one dimension a finite universe is just a circle. Thus, in two dimensions, one can regard the observed uniform expansion as the balloon's inflation; and two-dimensional galaxies, when they form, are spots on the balloon's surface.

A field of the type Turok considers is a vector, an arrow at each point of the Universe, with unit length and a direction that changes continuously from point to point. Two field configurations are said to have different texture if one configuration cannot be continuously changed into the other. It is easy to display what's going on in one dimension, so Fig. 2 shows two field configurations with different textures in a one-dimensional universe (a circle). In Fig. 2a, the arrows all point in the same direction, whereas in Fig. 2b, as one traverses the circle once in a clockwise direction the arrows also rotate clockwise by one revolution. The number of times the arrows rotate in one trip around the circle is called the winding number of the field configuration — this is zero for Fig. 2a and one for Fig. 2b. Any continuous deformation of the arrows, as in the sequence depicted in Fig. 3, preserves the winding number: one can deform the arrows to make the region where they wind as small as desired, but as long as the field is continuous and the length of the arrows is not zero, one cannot get rid of the twist. One can similarly visualize the evolution of texture for a two-dimensional

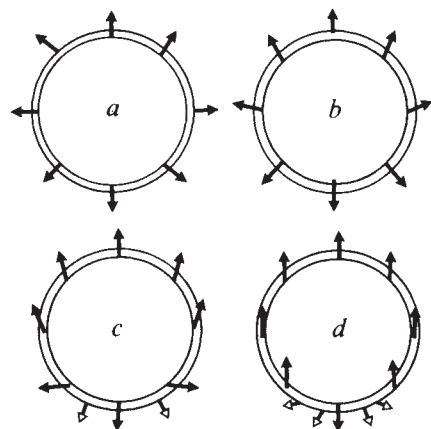
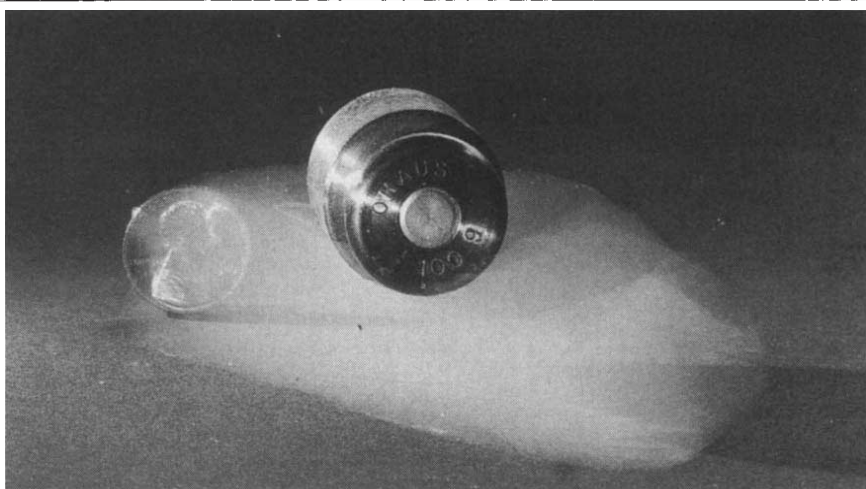


FIG. 3. A continuous sequence of Higgs field configurations, showing the contraction of texture with winding number one. *a*, The initial field configuration is deformed as the Higgs directions near the top of this one-dimensional universe start to fall, *b*, into alignment (it is energetically favourable for them to do so). *c*, Soon the Higgs field is aligned over most of the space, except for the 'knot' of texture developing at the bottom (which the grey arrows help to show). *d*, Finally most of the Universe has fallen into a state corresponding to winding number zero, but a small knot has been left at the bottom, which cannot be continuously got rid of without shrinking the field to zero.



'FROZEN SMOKE' is a term used to describe silica aerogels, a class of semi-transparent materials that includes some of the lightest known solids. Researchers at the Lawrence Livermore National Laboratory in California have developed a new method that enables them to make aerogels with a density as low as 5 mg cm^{-3} — ten times lighter than previously possible. The sample shown here, which weighs 0.06 g, is supporting about 1,600 times its own weight. A coin behind the material reveals its transparent quality. Researchers are considering aerogels as insulation, both in space vehicles and on Earth. They may also be useful in capturing cosmic dust for analysis without destroying or contaminating it. □

universe if one imagines that Figs 2 and 3 depict fields on a sphere instead of a circle: again, a field aligned in one direction has winding number zero, whereas a radical field has winding number one.

The field will evolve to the available configuration with lowest energy, and one lowers the energy by squeezing the twist into a smaller region. Although the energy density increases as the twist gets tighter, the total energy — (energy density) $\times$ (volume) — decreases as the volume goes to zero. Thus as a Universe with texture evolves, one expects the twists to collapse into small regions with high energy density, which Turok calls knots. Finally, when the energy density is extremely high, high enough to melt the Higgs field again, the field can unravel the knot — it can change its magnitude to zero and radiate the energy of the knot away in a spherical blast wave. Turok's suggestion is that objects ranging upward in size from galaxies to giant superclusters of galaxies might condense either about the knots or at intersections of blast waves from the annihilation of neighbouring knots.

Texture has been studied earlier in the context of a non-inflationary solution to the horizon problem^{6,7}. But it seems somewhat more artificial than strings, because texture occurs in a smaller set of theories: symmetry breaking more commonly leads to strings than to knots. (Knots require what is called global instead of local symmetry.) Strings, however, have been studied in some detail, and like all of the reasonably well understood models for the formation of large-scale structure, they have their problems. Among these is explaining the nonexistence of galaxies

with nuclei more massive than 4×10^{11} solar masses, when there is no known cutoff on the mass of string loops.

Alternatives to string models abound, exploiting the density perturbations expected to be present because of quantum fluctuations in the early Universe. However, these typically require that the stuff that condenses around the primordial density fluctuations be cold dark matter. Candidates for cold dark matter are undiscovered particles that appear in one or another theory of particle physics and would replace neutrinos as the main constituent of the Universe's mass. Cold dark matter has a relatively easy time forming galaxies, but a difficult time^{8,9} forming the larger scale structures — the beautiful filaments, voids, wall and great attractor¹⁰⁻¹³ seen in recent years. Global texture, if Turok's sketch of its primordial production and decay proves correct, is another chance to account for the structural complexity of our Universe. □

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1. Turok, N. *Phys. Rev. Lett.* **63**, 2625–2628 (1989).
2. Carswell, R.F. & Hewett, P.C. *Nature* **343**, 117–118 (1990).
3. Blumenthal, G.R. *et al.* *Nature* **311**, 517–525 (1984).
4. Zel'dovich, Ya, B. *Mon. Not. R. astr. Soc.* **192**, 663–667 (1980).
5. Vilenkin, A. *Phys. Rep.* **121**, 263–315 (1985).
6. Davis, R.C. *Phys. Rev. D* **36**, 997–999 (1987).
7. Kibble, T.W.B. *J. Phys. A* **9**, 1387–1398 (1976).
8. White, S.D.M. in *Inner Space/Outer Space* (eds Kolb, E.W. *et al.* 228–257 (Univ. Chicago, 1986).
9. Ostriker, J.P. & Suto, Y. *Astrophys. J.* **348**, 378–382 (1990).
10. Kirshner, R.F. *et al.* *Astrophys. J.* **248**, L57–L60 (1981).
11. de Lapparent, V., Geller, M. J. & Huchra, J. P. *Astrophys. J.* **302**, L1–L6 (1986).
12. Lynden-Bell, D. *et al.* *Astrophys. J.* **326**, 19–49 (1988).
13. Lilje, P.B., Yahil, A. & Jones, B. J. T. *Astrophys. J.* **307**, 91–96 (1986).

Tackling a loopy problem

Janet M. Thornton

WORK towards designer antibodies, which can be made to recognize a given target, has taken a step forward with two contributions to *Proceedings of the National Academy of Sciences*¹ and *Nature*². These papers describe approaches to modelling the three-dimensional structure of the antibody-combining site for a given amino-acid sequence of the hypervariable loops. Despite using rather different philosophies, both sets of authors have achieved good agreement between their models and experimentally observed structures; indeed, some loop backbone conformations can be modelled as accurately as they can be determined by medium-resolution X-ray crystallography. With the discovery of a new vector system allowing rapid production of antibodies in bacteria³, the stage is set for exploitation of antibody diversity and specificity.

The problem tackled by Martin *et al.*¹ and Chothia *et al.*² is a specialized application of building a model of a protein structure on the basis of the coordinates of a 'parent' structure (that is, one which is homologous in sequence). Studies of protein families for which several structures are known, including the globins⁴ and the serine proteinases⁵, show that the central 'core' of the family is well conserved whereas the loop regions vary considerably in length, sequence and conformation. The difficult part of modelling on the basis of homology is therefore to predict the loop regions correctly. For some structures, such as various enzymes, the loops may not be important for activity. But in the antibody structures, the six hypervariable loops form the 'recognition' site which allows the foreign molecule to be identified.

Since the first attempts at modelling⁶ there has been an increase both in the sophistication of the computational approach⁷⁻¹⁰ to modelling and in our understanding of what determines loop conformation^{11,12}. Loop regions have traditionally been described as the 'random-coil' connections between secondary structural elements. In modelling they tend to be defined slightly differently, as those residues which are not in the core or 'framework' (refs 5,11). For the immunoglobulins, the 'framework' region is defined in different ways by different groups. The original definition of the hypervariable loops¹³, based on sequence data alone, has been used by some groups^{14,15}. The alternative approach is to find a 'constant' structural framework by aligning the available structures in space and thereby defining the structurally conserved regions². Several groups have developed fully automated algorithms to

align structures and define the structurally conserved regions^{14,15}.

The problem is then reduced to modelling the loop regions. There are two basic methods — use of a knowledge base of available structures to provide the best possible loop conformation; or use of distance-geometry to generate all possible conformers, followed by choosing the best conformer on the basis of some type of 'energy' function. In a study of the serine proteinases, Greer⁵ was the first to show that the knowledge-based approach is surprisingly powerful. There are three obvious factors which combine to determine loop structure — the separation and orientation of the end-points where the loop joins onto the framework; the residue length and sequence of the loop; and interactions with the 'framework', other loop regions or both (context). Greer showed that loops from different members of the serine proteinase family could be successfully used to model a 'related' structure. Jones and Thirup⁷ later developed an elegant search routine to extract loops of a given length that satisfy the end-point separation and orientation. This method has been widely implemented and is successful because loops of the same length and end-point geometry but very different sequences often adopt the same structure.

An important proviso to this 'rule' has been further developed by Chothia and Lesk¹¹. By study of the hypervariable loops in the immunoglobulins they discovered that the loops appear to adopt a limited number of conformations which reflect the interaction of 'key' residues in the loop or framework. Previously it had been noted¹² that the conformations of short loops in β -hairpins were restricted and depended on critical glycine residues. Chothia and Lesk showed that five of the six hypervariable regions (excluding H3) adopt a small repertoire of main-chain conformations.

In their new paper², Chothia *et al.* provide additional support for their hypothesis by predicting and assessing the conformations of these five hypervariable regions in five new antibody structures. Perhaps more importantly, they analyse all known antibody sequences and suggest that 70–90 per cent of these sequences can be predicted on the basis of the current 'observed' repertoire. Obviously structures are not known for all these sequences. The hypothesis has been shown to be well founded, however, and although there may be one or two surprises I expect it will be proved broadly correct. But we are still left with 10–30 per cent of these five hypervariable regions, which cannot be

modelled from this knowledge base, plus the H3 loops.

Martin *et al.*¹ tackle the problem with a combination of the knowledge-based and *ab initio* methods. They used all the protein structures in the Brookhaven Databank, and for each hypervariable region looked for loops that have the requisite number of residues and satisfy a set of distance constraints. The distance constraints are derived from known antibody structures, although which structures and which constraints is not made clear. No sequence constraints are included at this point. They then delete five residues either at the distal end of the loop, or around a mutation, and search all conformational space using the program CONGEN⁹. This effectively allows the loop some 'freedom' to adjust to its environment. Loops of seven residues or less adopt a relatively restricted set of possible conformations, and a strictly knowledge-based or CONGEN approach is used. The set of possible conformations is then screened using a modified energy potential, and then a minimal solvent accessibility criterion for hydrophobic atoms; that is, the conformation having the lowest hydrophobic exposed area is selected from the five structures of lowest energy. The authors state that this method is "independent of decisions on the part of the user", which is a definite advantage.

How do the two methods compare? In fact, root-mean-square (r.m.s.) deviations from observed structures obtained using the two methods are not directly comparable. The groups model different length loops, use different atoms to calculate r.m.s. fit (not always explicitly stated) and Martin *et al.* model each loop in the presence of all the other 'correct' loops (and the antigen for HyHEL-5). This will inevitably improve the fit. Optimal deviations for main-chain atoms range from 0.3–1.4 Å in 19 loops predicted by Chothia *et al.*² in four different structures. For comparison, Martin *et al.* report values for backbone atoms of 0.6–1.4 Å for 12 loops in two different structures. Martin *et al.* also predict side-chain conformations which are crucial for defining the combining site topography. They achieve r.m.s. deviations for all atoms ranging from 1.4–2.8 Å for the 12 loops. Many side chains are located accurately, but a few aromatic rings are incorrectly orientated.

Both methods yield useful results, raising some interesting questions. Martin *et al.* use knowledge-based and *ab initio* information, which is clearly desirable and will be a requirement especially for unusual loops or for determining side-chain interactions where the current database is too limited. But the concept of key residues either in the loop or framework has been shown to be of crucial importance, and is not explicitly included

by Martin *et al.*. A logical development would be to create an automated algorithm which identifies these residues and uses the information in choosing between loops. Energy constraints and distance-geometry methods could then be used to optimize the loop structure. Conventional energy parameters have consistently been shown to be inadequate in identifying the native state. Consideration of the exposure of hydrophobic groups is a better discriminator^{16,17}.

Another question is whether it is always better to use loops extracted from the 'family' in preference to loops from non-related structures. Martin *et al.* employ non-antibody loops as templates in almost half of their predictions, but given the limited repertoire found by Chothia *et al.* it seems that, where applicable, antibody structures should be the 'preferred' knowledge-base. The answer to the question depends on the extent to which a loop structure is determined by its end-point separation and orientation rather than by interactions with the rest of the protein. All the antibody loops are built on the same framework in the same 'context'. Therefore it is arguable that these are the most appropriate. H3 loops apparently adopt many different structures, however, and for these and the other unusual loop sequences it will be necessary to

use a wider knowledge base.

Both papers make a clear contribution to our ability to model antibody loops and to the more general problem of modelling loops in homologous structures. Many laboratories are endeavouring to conquer this problem, not least to aid rational drug and vaccine design. It is a method with great potential which will, with some proteins, enable us to achieve that elusive goal of turning linear sequences into three-dimensional structures. □

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1. Martin, A. C. R., Cheetham, J. C. & Rees, A. R. *Proc. natn. Acad. Sci. U.S.A.* **86**, 9268–9272 (1989).
2. Chothia, C. *et al.* *Nature* **342**, 877–883 (1989).
3. Huse, W. D. *et al.* *Science* **246**, 1275–1281 (1989).
4. Lesk, A. M. & Chothia, C. *J. molec. Biol.* **136**, 225 (1980).
5. Greer, J. J. *J. molec. Biol.* **155**, 1027–1042 (1981).
6. Browne, W. J. *J. molec. Biol.* **42**, 65–86 (1989).
7. Jones, T. A. & Thirup, S. *EMBO J.* **5**, 819–822 (1986).
8. Blundell, T. L. *et al.* *Nature* **326**, 347–352 (1987).
9. Bruccoleri, R. E., Haber, E. & Novotný, J. *Nature* **335**, 564–568 (1988).
10. Fine, R. M. *et al.* *Proteins* **1**, 342–362 (1986).
11. Chothia, C. & Lesk, A. M. *J. molec. Biol.* **196**, 901–917 (1987).
12. Sibanda, B. L. & Thornton, J. M. *Nature* **316**, 170 (1985).
13. Wu, T. T. & Kabat, E. A. *J. exp. Med.* **132**, 211–250 (1970).
14. Orengo, C. & Taylor, W. R. *J. molec. Biol.* **208**, 1–22 (1989).
15. Sali, A. & Blundell, T. L. *J. molec. Biol.* (in the press).
16. Novotný, J. *et al.* *Proteins* **4**, 19–30 (1988).
17. Barmann, G. *et al.* *Prot. Eng.* **2**, 329–334 (1989).

MOLECULAR GENETICS

Colorectal cancer genes

Ellen Solomon

COLORRECTAL carcinomas are among the most common malignancies in man. A long-standing model of this disease is that tumours arise from benign adenomatous polyps, which then progress to adenocarcinomas through many mutational steps. In a small subset of cases, about 8 per cent, susceptibility to colorectal tumours is clearly inherited; these tumours fall into distinct clinical categories, including adenomatous polyposis coli (APC) and the hereditary non-polyposis colon cancer syndromes (HNPCC). The inherited diseases provide an enormously valuable tool for identifying at least some of the colorectal cancer genes. In the past few years there has been considerable progress in the localization, identification and isolation of three colorectal cancer genes; in confirming the large number of mutational events in the progression from adenoma to carcinoma; and in revealing that, at the cellular level, there is probably little to distinguish the mutations in inherited and sporadic disease. At a meeting on hereditary colorectal cancer*, many findings were brought together and some important results were reported, including the iso-

lation of a new colorectal cancer gene.

Before 'reverse genetic' approaches could be used to isolate colon cancer genes, the genes had to be approximately located on their chromosomes. In the case of familial disease this could be achieved by linkage analysis, as was successfully done for APC and a locus on chromosome 5 at 5q15–q22 (refs 1,2). With sporadic tumours, family studies are not possible so that analysis of allele loss, with the aim of identifying tumour suppressor genes, has been the main alternative strategy. This approach has been particularly fruitful with colorectal cancer, and the power of the method in identifying small chromosomal regions containing tumour-related genes has been extremely impressive. Allele loss on a large number of chromosomes has been reported by many different groups (for review of this literature see ref. 3), including chromosomes 5, 17, 18, 22, 6, 1, 8 and 9, and it is clear that the progression to adenocarcinomas involves at least eight mutational events. These events need not be the same in all cases, nor need they occur in the same order. Attempts to determine a temporal sequence for the accumulation of mutations has produced only a rather general

picture for a few of the events⁴, and it seems that it is the total accumulation of mutations which results in the carcinoma, and that several different pathways may be involved. The chromosomes that have been subject to particular attention are 5 (because of the APC gene), and 17 and 18 (because of the large extent to which they show allele loss). In the current working model⁵, a first mutation (whether germline or somatic) in the APC gene on chromosome 5 is believed to be an early event in adenoma formation, and is probably specific to colorectal cancer. Loss of the second allele is a later event, occurring nearer the time of carcinoma transition. K-ras mutations are not evident in early adenomas, but are seen in larger adenomas and in general follow loss on chromosome 5. Some allele loss on chromosomes 17 and 18 is found in larger adenomas, but it increases dramatically in carcinomas; these losses are believed to be relatively late events in the progression, with loss on 18 preceding that on 17 (refs 4,5).

A major new finding announced at the meeting, now published⁶, was the isolation and identification of the gene on chromosome 18q (B. Vogelstein, Johns Hopkins Oncology Center, Baltimore). The region with the highest allele loss, and thus that most likely to contain a colorectal cancer gene, is 18q21–q22. Use of probes in this region on tumour material revealed two tumours with abnormal pattern, one with a homozygous deletion and the other with an altered band. The latter band was cloned and a G-to-A mutation found, not in a coding region, but in a position suggesting a splice-site mutation. Following a 400-kilobase (kb) walk, isolation of conserved regions, sequencing, identification of exons and amplification of messenger RNA using the polymerase chain reaction, Vogelstein's group isolated a 2–3-megabase gene with a 12-kb mRNA. The gene, called DCC (deleted in colon cancer), is expressed in normal colonic mucosa but usually not in tumours. It seems to be a hotspot for somatic mutations in 12 tumours examined, and is proposed as a candidate colorectal tumour suppressor gene. The specificity of loss of this allele to colorectal tumours is not yet known; but because it comes late in the sequence, the suppressor activity might be a general one affecting tumour growth in which case mutations would be seen in other tumours. Of great interest is the fact that the gene product is homologous to cell-surface adhesion molecules, such as neural cell-adhesion molecule (NCAM) and contactin, and that like them it contains four IgG and three intermediate filament domains. This type of molecule is likely to have an important role in tumour growth, and the gene provides an excellent system with which to pursue the biology involved.

The short arm of chromosome 17 also

*Fourth International Symposium on Colorectal Cancer, Kobe, Japan, 9–11 November 1989.

has extremely high allele loss not only in colorectal carcinomas but also in most adult tumours, including those of breast, brain, lung, bladder, ovaries, cervix, adrenal cortex and bone (see ref. 7). In the small chromosomal region of maximum allele loss at 17q13.1 is the gene encoding p53. Although initial studies showed no gross rearrangements either in this gene or in its mRNA, Baker *et al.* have suggested that this is indeed the "colon cancer locus", as two tumours exhibiting allele loss had sustained point mutations in the remaining allele⁸. More tumours have now been investigated by this group and others, and most tumours exhibiting allele loss have missense mutations in the remaining p53 gene⁷. This was the first tumour suppressor gene for colorectal cancer to be isolated, and it has allowed two extremely important questions to be addressed — whether these genes necessarily behave in a recessive manner⁹, and whether allele loss is a second event. The p53 gene has been primarily studied in rodent systems where mutant p53 gene, in concert with activated *ras*, transforms primary rat embryo fibroblasts (for review see ref. 5). The mutant genes act dominantly, so that transformation occurs even in the presence of the wild-type allele.

But loss of the wild-type protein has a potentiating effect, which suggests first that the wild-type protein behaves as a tumour suppressor, and second that the mutant and wild-type protein interact, perhaps, as has been suggested, by the oligomerization of the proteins. If the mode of action in the rodent systems holds in human tumour formation, a first mutation, seemingly a point mutation, would confer growth advantage upon the cell, and would be acting in a dominant negative manner rather than in the predicted recessive one. Loss of the wild-type allele could occur later in tumour formation, giving further growth advantage to the cell. If this is the case, it would be expected that those tumours not yet showing allele loss would still have a mutation in one allele. Such tumours have indeed been found, confirming that allele loss is a second event. Mutations in the p53 gene have now also been identified in brain, breast, lung and mesenchymal tumours, showing that this is an extremely important gene in tumour growth⁷.

Allele loss on chromosome 5, unlike that on chromosome 17, seems to be specific to colorectal tumours. In sporadic, or non-inherited, tumours, loss occurs principally in the 5q15–q22 region, with a peak of loss at 60 per cent (A. H. Wyllie, University Medical School, Edinburgh), strongly indicating that the loss is occurring at the APC gene. Allele loss is also seen in larger sporadic adenomas⁴. Until now, there have been few data on allele loss in carcinomas from APC patients because this material is not commonly

Finding space for granite intrusions



WHERE does the space in the Earth's crust that intrusive granite bodies occupy come from? This problem has long puzzled geologists. Evidence that the intrusions push aside the intruded rocks is usually absent or feeble; flexuring is rarely adequate to provide all the space required. Pieces of host rock partially prised off the roof indicate that blocks could become detached and sink through the rising granite magma. But it is hard to see how the magma could remain molten while a shower of cold blocks sank through it. And anyway, the lack of exposed bases of granite bodies — we usually see the tops or sides — exacerbates the conundrum.

On page 452 of this issue, D.H.W. Hutton and co-workers report their discovery of a remarkably well-exposed granite in Greenland in which both the top and the bottom are exposed. As shown in the photograph, there is a dark band of granite sandwiched between the lighter coloured rocks above and below, towards the top of the mountain. The disposition and structure of the band

show that the granite was intruded syn-tectonically, filling the space created by ductile extension along a shear zone created by the separation of crust along an older, gently inclined discontinuity. It is now widely recognized that the pulling apart and extension (by thinning) of crust



is a common occurrence, so that this discovery probably applies elsewhere. It complements a previous observation, also by Hutton, that spaces open by horizontal movement along vertical faults. Perhaps the largest intrusions, which obviously need the most room, simply filled holes created as the crust was pulled apart.

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available in Western Europe or the United States owing to screening and prophylactic removal of the colon. A Japanese study on a large sample of APC adenomas as well as carcinomas (M. Miyaki, Tokyo Metropolitan Institute of Medical Science) confirmed previous results that allele loss does not occur in the APC adenomas¹⁰ and dramatically showed that, in APC carcinomas, allele loss from chromosome 5 occurs at the same rate as in the common sporadic carcinomas.

This exciting result neatly ties together a model for the role of the APC gene, along the lines originally predicted by Knudson⁹. In APC patients a single mutation in one allele is inherited, and this mutation is sufficient for adenoma forma-

tion to occur. Again, this gene is clearly not acting recessively at the cellular level. In the progression to carcinoma, loss of the second allele confers full growth advantage. In sporadic cases, the model suggests that a somatic mutation in the APC gene (equivalent to the inherited mutation) occurs early in adenoma formation, and that loss of the second allele, observed later in the progression towards carcinoma formation, is essentially the same event as occurs in the APC tumours. The model cannot be tested fully, however, until this gene is isolated, and the proposed first mutational event can be observed. This same Japanese group was also able to test the model with a carcinoma from an APC individual, in which

as would be predicted, the chromosome 5 allele that was lost from the normal parent, and the allele that was retained was from the affected parent.

The APC gene awaits isolation. The region has been further defined genetically (C. Tops, University of Leiden; Y. Nakamura, Tokyo Cancer Institute), physically (Nakamura; A.-M. Frischau, ICRF, London) and by chromosomal deletions from patients with APC¹¹. A number of new cases with mental retardation and APC was reported (M. Chiba, University of Akita, Japan) although no deletions were karyotypically visible. These and previously reported cases suggest that a gene involved in mental retardation may be within a small distance of the APC gene. Those working on the isolation of the gene assume that, because the new mutation rate is high, the gene may be large and many different types of mutation may exist, and that these will be readily detectable once the region is covered with enough probes. The discovery of closely linked markers flanking APC has allowed the markers to be used in premorbid diagnosis of this disease. The majority of families will now be informative and predictions can be made with greater than 95 per cent confidence as to whether an individual has inherited the disease or not. This is a vast improvement on the situation where an individual with a one in two risk of developing the disease had to be screened annually by sigmoidoscopy, from the age of about 14 until about 35, for the presence of colonic adenomas. For example, an individual with a prior probability of one in two and a negative sigmoidoscopy at age ten has a 49 per cent chance of developing the disease. Now this same individual with negative linkage has this chance reduced to about 5 per cent (J. Slack, Royal Free Hospital, London; G. M. Petersen, Cedars-Sinai Medical Center, Los Angeles).

As yet, positive genetic linkage has been demonstrated for only one of the hereditary non-polyposis colon cancer syndromes. The cancer family syndrome (Lynch syndrome II) has been provisionally linked to the Kidd blood group on chromosome 18 (ref. 12), an intriguing assignment in view of the location of DCC gene on this chromosome. Linkage to the

same markers as APC on chromosome 5 was reported for an interesting family from Utah, K353, with features of APC but with far fewer adenomas (R. L. White, Howard Hughes Medical Institute, Salt Lake City). This variation in phenotypic expression might be expected for a gene with many different types of new mutations. That this is the case is also borne out by the observation that Gardner's syndrome, which is similar to APC but has other extra-colonic manifestations, is allelic to APC. There is also a possibility that phenotypically milder APC gene mutations occur, and that low penetrance alleles are even responsible for some of

the increased risk of colorectal cancer in first-degree relatives of patients with apparently sporadic colorectal cancer¹³ (T. Bishop, ICRF, Leeds; R. W. Burt, University of Utah, Salt Lake City). The isolation of this gene and analysis of its mutations is now crucial not just for further investigation of APC, but for the molecular understanding — and perhaps screening, management and eventually treatment — of common colorectal cancer. □

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PALAEOECOLOGY

Ups and downs in the Sahel

Peter D. Moore

THE Sahel region of Africa, a strip stretching from Senegal and Mauritania in the west to the Sudan in the east, forms the southern edge of the Sahara Desert. The steepness of its climatic gradient (with annual rainfall decreasing by about 1 mm with every 1.4 kilometre one travels north) means that the area is particularly sensitive even to small climatic shifts. It is natural, therefore, that detailed information is sought on the recent climatic history of the Sahel region to establish a basis for future climatic prediction in a greenhouse world. The recent work on climatic changes in West Africa by Lézine and Casanova¹, and the collation and discussion of Sahelian work to date by Lézine², demonstrate that there have been substantial shifts in the vegetation zones of the area over the past 20,000 years.

The sources of evidence for past climatic changes in this currently semi-arid region are various. The closest to documentary evidence of former abundance of moisture are rock paintings that depict a succession of scenes, from game-rich savanna, to cattle-herding pastoralists, to desert³. Geomorphological and palaeontological evidence comes in the form of fossil river systems and drainage networks, such as the Wadi Howar system in the Sudan⁴ which contains fossil remains of large river bivalves, hippopotamus and crocodile in a region currently receiving only 25 mm of rain a year. Past vegetation has left its signature in the pollen-containing sediments of former lakes in the Sahel, such as the Selima Oasis and El Atrun in the northern Sudan⁵, which provide evidence of former savanna grasslands in areas now hyper-arid. There are also relict patches of vegetation that have been isolated far to the north of the vegetation belts to which they belong. These are presumed to represent the residue of a former, more northerly extension of these zones.

Information from botanical and palaeoecological studies is more abundant in eastern and central Africa than in the west, but Lézine and her colleagues have helped to rectify this imbalance. She has found relatively ancient polleniferous deposits in some unlikely sites, such as the small mires between dunes on the Atlantic coast of Africa, and similar interdune depressions inland in Mauritania. One advantage of using small sites of this type is that they have a relatively local pollen catchment, and are not quite as subject to the problems of interpretation as are large basins to which there has been both alluvial and aerial input of pollen (a line of reasoning also gaining much ground in temperate palynological studies⁶).

From a scattered assemblage of pollen data, Lézine has now collated information covering the full length of the Sahelian strip². She concludes that 18,000 years ago, when the glaciers had reached their maximum extent in the high latitudes, the savanna grasslands with *Acacia* trees grew only as far north as 10° N, considerably to the south of their current position. But at 8500 years BP (before present) there was a concerted and rapid northward extension of moisture-demanding vegetation types, eventually reaching some 400–500 km north of their current boundaries. The reversal began about 6100 BP and intensified around 4500 BP, but it was about 2,000 years ago when the semi-arid steppe vegetation finally became established in the zone between 13° N and 19° N, where it lies today.

Lézine interprets these palaeobotanical data in terms of changing precipitation; in the early Holocene, annual rainfall in the Sahel was about 300 mm more than it is now. The gradual southward movement of vegetation belts after 4500 BP may represent the establishment of a more arid dry season between the monsoon rains. The work of Lézine in West Africa has

1. Bodmer, W. F. *et al.* *Nature* **328**, 614–616 (1987).
2. Leppert, M. *et al.* *Science* **238**, 1411–1413 (1987).
3. Vogelstein, B. *et al.* *Science* **244**, 207–211 (1989).
4. Vogelstein, B. *et al.* *New Engl. J. Med.* **329**, 525 (1988).
5. Cavanee, W. K., Hastie, N. D., & Stanbridge, E. J. (eds) *Current Communications in Molecular Biology: Recesive Oncogenes and Tumour Suppression* (Cold Spring Harbor Press, New York, 1989).
6. Fearon, E. R. *et al.* *Science* **247**, 49–56 (1990).
7. Nigro, J. M. *et al.* *Nature* **342**, 705–708 (1989).
8. Baker, S. J. *et al.* *Science* **244**, 217–221 (1989).
9. Knudson, A. *Cancer Res.* **45**, 1437–1443 (1985).
10. Solomon, E. *et al.* *Nature* **328**, 616–619 (1987).
11. Varescol, L. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **86**, 10118–10122 (1989).
12. Boman, B. M., Lynch, H. T., Kimberling, W. J. & Wildrick, D. M. *Cancer Genet. Cytogenet.* **34**, 153–154 (1988).
13. Cannon-Albright, L., Skolnick, M., Bishop, D. T., Lee, R. & Burt, R. *New Engl. J. Med.* **319**, 533–537 (1988).

thus confirmed the findings of Ritchie and Haynes in the Selima Oasis of the Sudan⁵, and has demonstrated that the north-south migration of vegetation belts during the Holocene has been a feature of the entire 6,500-km length of the Sahel.

Also interesting is the recent analysis of a rich assemblage of wetland animal fossils discovered in the eastern Sahara⁷, which has shown that the moist conditions typifying the early part of the Holocene were matched, or even exceeded, in that area during the last interglacial some 135,000 years ago. The climatic fluctuations in the

Sahel, which produce such distressing social consequences, have evidently been proceeding for a very long time. □

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1. Lézine, A.M. & Casanova, J. *Quat. Sci. Rev.* **8**, 45–55 (1989).
2. Lézine, A.M. *Quat. Res.* **32**, 317–334 (1989).
3. Riset, J.P. *Endeavour* **8**, 75–84 (1984).
4. Pachur, H. J. & Kropelin, S. *Science* **237**, 298–300 (1987).
5. Ritchie, J.C. & Haynes, C.V. *Nature* **330**, 645–647 (1987).
6. Moore, P.D. *Nature* **311**, 412 (1984).
7. Kowalski, K. et al. *Quat. Res.* **32**, 335–341 (1989).

MICROELECTRONICS

Electron optics for transistors

S. Washburn

THE transistor has revolutionized the electronics industry and, because of its small size, it is the *sine qua non* of modern computers. The improvement of computing power and speed relies mainly on making smaller and faster transistors. Although conventional transistors, relying on classical behaviour of electrons, can be still smaller than they are today, a completely new approach has come to light which employs the wave mechanics of the electrons. Several groups are investigating transistors that rely on the manipulation of beams of electrons by methods similar to classical optics^{1–4}. This new species of transistor might eventually become faster and more efficient than any available now.

The analogy between electron and photon propagation (classical wave optics) has long been used. At the fundamental level, standard imaging instruments like the scanning electron microscope rely on the same principles employed to make light microscopes. Electrons, however, are susceptible to more kinds of forces than the photons in a beam of light. Their electrical charge means that they can be deflected in a magnetic field, for example, by the

Lorentz force, which electrically neutral photons ignore. Electron-optical devices tend to be rather cumbersome, however, mainly because of the high voltages required to generate and accelerate energetic electron beams and because of the elaborate vacuum systems required to provide empty space for the beams to propagate through (any residual gases disperse the electron beam through collisions).

In the past few years, however, it has become increasingly clear that solid-state systems can be manufactured in which the electrons travel freely, or 'ballistically', for appreciable distances. Inferring from the resistivity of the material, one frequently finds that the electrons' mean free path is several micrometres long, or more, so that rather large distances are traversed between successive collisions of the electrons with crystal impurities. Although the distances are not as large as in an ultra-high vacuum, the technology for steering and detecting the beam in the solid-state devices is not nearly so demanding, so that this too can be fitted into the short ballistic paths: the scales are limited by modern circuit lithography, routinely extended down to sub-micrometre lengths. The large mean free path provides for the possibility of studying electron optics in solid-state devices and of making ballistic transistors based on the wave mechanics of the electrons.

Conventional transistors dissipate a good deal of energy and, when packed sufficiently densely to make, for example, a computer memory, require elaborate cooling methods to keep them from overheating. In contrast, ballistic transistors would operate at much lower temperatures — certainly at 77 K (in a bath of liquid nitrogen) and possibly lower. Although the refrigeration would still need to be fairly elaborate, the smaller dimensions should make it possible (but by no means certain) that such ballistic circuits could operate more quickly and also dissipate much less power than

conventional room-temperature devices.

The essential feature of optics is the focusing of a beam. In classical optics this is accomplished by a convex lens which bends the incident beam of light as described by Snell's law for refraction. The focusing of electrons can be accomplished by one (or a combination) of several forces. Electrostatic forces are sometimes used in the conventional electron microscope: passing the beam between a pair of electrodes connected to positive and negative voltages bends the beam towards the positive pole. The same scheme can be applied to the solid-state case as well. Another more elegant method of focusing has been used by van Houten *et al.*¹. In their experiment, the electrons emerge from a narrow constriction (a point contact) which serves as a collimating injector for the current. The electrons' path is then bent by the Lorentz force from a magnetic field applied perpendicular to the plane of the device. The resulting trajectories are circular cyclotron orbits with diameters inversely proportional to the applied magnetic field. If the electrons are detected at another point contact, then whenever an integer number of these 'Landau' diameters matches the distance to the detector, there is a peak in the signal (Fig. 1).

The use of a magnetic field to focus the electrons is, however, rather cumbersome, because the transistor would have to be imbedded in a solenoid which would then have to be controlled to 'switch' the transistor. More convenient is the use of a gate (which changes the electron energy, wavelength and number density) in some region of the sample. This method is already used in conventional transistors. The attractiveness and simplicity of this technique for ballistic devices have been discussed in two recent publications. New theoretical papers by de Raedt, García and Sáenz², and Heiblum and Fischetti³, suggest that the gate can focus by being an energy selector. By directly solving the Schrödinger equation for the geometry in Fig. 2, de Raedt, García and Sáenz find

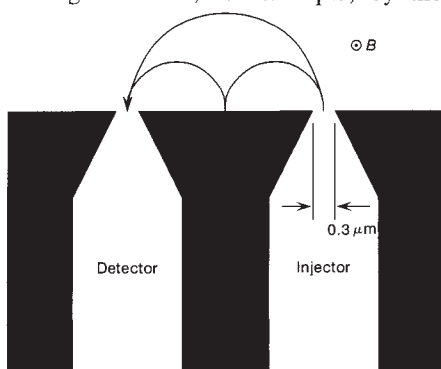


FIG. 1 Focusing by a magnetic field B (directed out of the paper) of electrons emitted from the right-hand point contact onto the left-hand point contact occurs when an integral number of 'Landau' orbits fit exactly onto the separation between the two contacts. Transistor switching could be driven by varying the applied field strength.

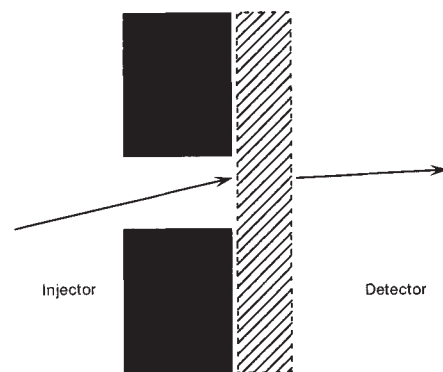


FIG. 2 The device proposed by de Raedt, García and Sáenz comprises a point contact followed by tunnelling barrier draw up by a gate. Electrons entering from all angles on the left are focused onto the axis of the device when they emerge to the right of the gate.

that a negative gate voltage substantially reduces the angular spread of electrons emerging from the point contact. The use of gates as energy selectors (or spectrometers) has been demonstrated experimentally; in one case, gates were used to make a ballistic, hot-electron transistor with considerable gain that operates at liquid-helium temperature⁵. In the proposed focusing transistor, after electrons emerge from a point contact, the gate restricts which of them travel into the detector on the right. The optimum focusing of the gate occurs when the electrons draw up a potential barrier that exceeds their energy, so that they are transported across the device by tunnelling through the barrier.

The analysis of the proposed tunnelling transistor ignores one salient feature of the physics: the authors assume that the walls of the point contact are perfectly square. In practice, the corners of any real device will be rounded somewhat (either

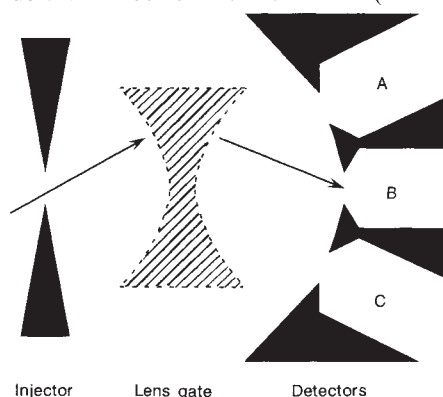


FIG. 3 The electron-lens transistor⁴ works in simple analogy with classical optics to focus electrons from the point contact on the left into a particular contact (B) on the right.

because of imperfect lithography or because the electrons themselves will screen the large electric fields at the corners), so that the incoming electrons tend to see barriers with round corners. This rounding leads to an adiabatic coupling of the transport modes in the narrow region to those in the injector⁶ and to an extra collimation of the incoming beam^{7,8}, so that even in the absence of the tunnelling gate, the outgoing beam tends to lie along the axis of the device. This collimation was recently confirmed in an experiment by Molenkamp *et al.*⁹. The proposed transistor also has another drawback. To be of practical use, it must be able to carry a substantial current, but the use of tunnelling to focus the electrons places severe limits on the throughput. Most of the input current is reflected back into the injector by the gate.

An ingenious approach to focusing without severely limiting the current has been devised by Sivan, Heiblum, Umbach and Shtrikman⁷, who use a classical lens to focus the electrons injected through a point contact as depicted in Fig. 3. This

method does not require tunnelling through the lens, but relies instead on simply changing the electron wavelength in the region under the gate. This refracts the beam of electrons just as a bit of curved glass refracts a beam of light. In the case of electrons, the focusing lens is convex and not concave as is the lens for light. The essential reason for the difference is that electrons in the gated region have lower energy and therefore longer wavelength, whereas the energy (and therefore the frequency) of the photons is fixed so that moving more slowly in the denser medium, they have shorter wavelength. The result is that whereas a convex lens is required to focus a columnar beam of light to a point, to focus the electron beam, one must use the opposite curvature — a concave lens. One can also apply a perpendicular magnetic field to such a transistor to guide the beam into one of the other contacts. For instance, a small field out of the page will push the focused beam into contact A, whereas reversing the field would focus the beam at C.

The practical usefulness of any of these devices is still very much in doubt. For logic circuits, the output of one transistor must be able to drive the gate of the next (and preferably several at once). The voltage scales in the focusing devices are off by orders of magnitude from achieving this goal. Typical gate voltages are 100 mV, but typical output voltages^{4,10} are a few microvolts. To conquer this disparity, the transistors would have to operate under very extreme conditions: extremely low carrier density, for instance. Under such stringent conditions, the fraction of such critically adjusted devices that actually work will be very small. Even if there were a successful yield, then the very small width reduces again the available current flow. In short, because of these and other technical problems¹¹, it seems unlikely that, in the near future, these transistors can be made to perform useful functions as amplifiers or logic switches. □

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1. van Houten, H. *et al.* *Phys. Rev.* **B39**, 8556–8575 (1989).
2. de Raedt, H., García, N. & Sáenz, J.J. *Phys. Rev. Lett.* **63**, 2260–2263 (1989).
3. Heiblum, M. & Fischetti, M.V. in *Topics in Current Physics* (ed. Capasso, F.) (Springer, Berlin, in the press).
4. Sivan, U., Heiblum, M., Umbach, C.P. & Shtrikman, H. *Phys. Rev. Lett.* (submitted).
5. Palevski, A., Umbach, C.P. & Heiblum, M. *Appl. Phys. Lett.* **55**, 1421–1423 (1989).
6. Glazman, L.I., Lesovik, G.B., Khmelnitsky, D.E. & Schecter, R.J. *JETP Lett.* **48**, 238–241 (1988).
7. Beenakker, C.W.J. & van Houten, H. *Phys. Rev.* **B39**, 10445–10448 (1989).
8. Baranger, H.U. & Stone, A.D. *Phys. Rev. Lett.* **63**, 414–417 (1989).
9. Molenkamp, L.W. *et al.* *Phys. Rev.* **B41**, 1247 (1990).
10. Spector, J.S. *et al.* *Pap. presented at Symp. New Phenom. Mesoscopic Structures* (Kona, Hawaii, 1989).
11. Landauer, R. *Phys. Today* **42** No. 10, 119–121 (1989).

Life in reverse

THE basic molecules of life all have a specific handedness. Its amino acids are all left-handed, whereas its sugars are right-handed. Molecules of opposite handedness can be synthesized, but are biochemically useless, or even poisonous.

The very first life forms may well have had all sorts of biochemical chiralities; but the ensuing 'war' between them was decisively won by the present arrangement. A biochemically 'inverted' animal, if one could be created, would be quite unable to digest any food it could find in our world. An inverted plant, however, is quite feasible. Plants build themselves entirely from small molecules like water and carbon dioxide, which have no handedness. So Daedalus is now trying to create the world's first inverted plant.

His starting point is a minute exception to the general rule. The micro-organism *Bacillus licheniformis* weaves its cell wall from right-handed amino acids in an effort to make itself indigestible to phagocytes. DREADCO's biochemists are intrigued: surely it cannot have mirror-image DNA? More likely some small 'switch' in DNA determines the handedness of its products, and *B. licheniformis* has ingeniously set this switch oppositely to everyone else. The DREADCO team hopes to extract the switch by standard genetic engineering methods, and to insert it into various promising plants.

The most promising, from Daedalus's point of view, are the structural and textile plants like oak, pine, flax, cotton and so on. They would be immune to wood borers and boll weevils; and would yield timber, paper and textiles which would magically resist rot and decay. Inverted timber would revolutionize the construction industry, which in rain-soaked Britain is paranoid about rot and fungal attack. Inverted wallpaper would never go mouldy, and inverted clothing would be moth proof.

Another DREADCO goal is inverted vegetables. They may taste a bit strange (some chiral molecules taste differently to their mirror images), but with luck they will be harmless and quite acceptable to the human palate. To the human stomach, however, they will be untouchable and totally devoid of nourishment. Inverted lentils, nuts and brown rice, combining vegetarian rectitude with zero delivered calories, would be an ideal slimming diet for earnest macrobiotic folk.

A stand or field of the new plants will rapidly get cluttered up with decay-proof stalks, leaves, fallen timber, and so on. Perhaps *B. licheniformis* might mutate to exploit this residue, starting a closed local 'inverted ecology'. Its competition with the surrounding normal ecology might mimic the primeval war of the chiralities most instructively.

David Jones

Coral bleaching in Jamaica

SIR—Since early October 1989, corals in Jamaican reefs have begun to lose their pigmentation (bleach). As in the 1987–88 bleaching event in the Caribbean¹, all varieties of reef-building corals, ranging from the surface to more than 30 m depth, are affected. Repeated dives along the north coast of Jamaica and reports from divers indicate that the phenomenon is widespread, and that it began no earlier than late September.

As in the earlier event, bleached corals are common in all fore-reef waters, are relatively uncommon in the back-reef and are not confined to areas of unusual local environmental stress². No new bleaching occurred in Jamaica in 1988, except for reefs just downcurrent from major river inflows, which were heavily bleached for about a month after large amounts of muddy freshwater runoff were dumped on them by hurricane Gilbert. Areas affected by the hurricane and those untouched by it are now bleaching.

Although the bleaching event still seems to be in its early stages, the overwhelming majority of corals are already affected. Many species have already completely lost their pigmentation, leaving areas of bleached white skeleton or tissue (for example *Agaricia*, *Millepora* and *Palythoa* species), some have preserved secondary screening pigments which make the colony appear unusual blue, green or pink colours (such as *Siderastrea*), whereas others have become very pale (such as most *Montastrea*, *Diploria*, *Porites*, *Eusmilia*, *Dendrogyra*, *Meandrina* and *Erythropodium*). Among the reef-building corals, only *Madracis* and *Acropora* seem minimally affected. Although the intensity of bleaching has not yet reached the greatest levels reached in late 1987, this year's event seems to be more widespread: *Montastrea annularis* colonies that bleached in 1987–88 are bleaching again, accompanied by pigment loss in colonies unaffected by the previous event. Many Jamaican genera (such as *Agaricia*, *Siderastrea*, *Porites* and *Diploria*) are much more strongly affected than by the previous episode.

As yet, there are no reports of bleaching elsewhere in the Caribbean (E. Williams, personal communication). It will be important to document whether the phenomenon is occurring in other parts of the region, as well as its onset and duration. Information should be sent to Dr Ernest Williams, Caribbean Aquatic Animal Health Project, Departamento de Ciencias Marinas, Universidad de Puerto Rico, PO Box 5000, Mayaguez, Puerto Rico, 00709.

The 1987–88 bleaching event began in late July, reached its peak around December and was largely complete by

March 1988 (ref. 2). Maximum seawater temperatures in Jamaica are generally reached in October or November, but in 1987 temperatures rose early, and did not begin to fall until December. In October 1989, ocean temperatures along the north coast of Jamaica were above 30 °C, and were at this level since at least early August. If bleaching is linked to a physiological reaction of corals and their symbiotic algae to elevated sea temperatures^{3,4}, the phenomenon could continue for at least a further month or two, depending on the time course of heat storage in Caribbean waters.

The 1987–88 bleaching episode raised concern of possible linkages between coral bleaching, rising tropical sea surface temperatures and the greenhouse effect, but its failure to appear in 1988–89 allowed the phenomenon to disappear from public awareness. Although it is too early to predict the ultimate severity or duration of the 1989 episode, there should be serious concern about its regional economic effects and global environmental implications.

During the nine months of the 1987–88 bleaching event, Caribbean reef-building

corals failed to grow². Repetition of such events will alter the food chain of the coral-reef ecosystem, making corals less able to compete with rapid growth of fleshy algae. They could ultimately convert coral reefs into algal habitats, with severe economic losses from deterioration of reef fisheries, tourism and shore protection. Recent information suggests that the 1989–90 event has peaked and is concentrated in Jamaica. (Details of the distribution and course of the event are in preparation, Caribbean Research Community.)

Because Caribbean coral bleaching may be a visible warning of the early stages of global warming, there is a case for building now the means for monitoring the phenomenon of coral bleaching and the research capability for understanding its causes.

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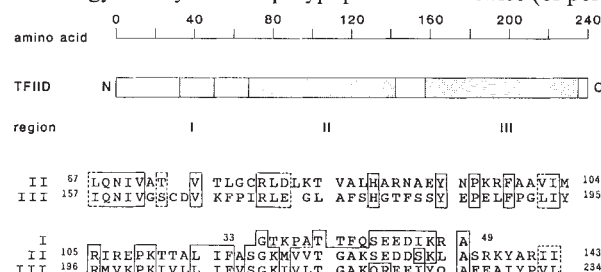
1. Williams, E. H., Goenaga, C. & Vicente, V. *Science* **238**, 877–878 (1987).
2. Goreau, T. J. & Macfarlane, A. H. *Coral Reefs* (in the press).
3. Hayes, R. L. *Coral Reefs* (in the press).
4. Sandeman, I. *Proc. Ass. Island Marine Labs. Carib.* **21**, 50 (1988).

Cryptic initiation sequence revealed

SIR—Several groups^{1–3} have recently cloned the gene encoding the protein binding to the TATA box and known as TFIID. This protein plays a key role in the initiation of transcription by polymerase II by binding specifically to the canonical AT-rich element found in the promoter region of many eukaryotic genes. The cascade of reactions triggered by binding of TFIID to the TATA box ensures the accurate start of the transcription process.

The nucleotide sequence of the TFIID gene corresponds to a protein of 240 amino acids with no strong sequence homology to any known polypeptide and

But careful inspection of the TFIID amino-acid sequence reveals that a major portion of the protein is made up of two segments with clear homology with each other. Direct alignment (see figure) and matrix comparison (data not shown) show that the region from amino acid 67 to 143 (II in the figure) is very similar to the region (III in the figure) from amino acid 157 to 234 (35 per cent identity, 47 per cent strong homology). Moreover, the C-terminal regions of these repeats contains a region (amino acids 121 to 135 and amino acids 212 to 226) having less obvious but still significant homology with the N-terminal stretch of amino acids 33 to 49 (I in the figure). Thus it seems that TFIID consists of a polypeptide repeated twice (or perhaps two and a half times).



Alignment of TFIID regions. Three portions of the predicted amino-acid sequence of TFIID, using the single-letter amino acid code. Identical amino acids are boxed; those with closely similar physico-chemical properties (K,R; D,E; T,S; and V, I and L) are shown in dashed boxes.

in which there are no obvious functional domains or highly conserved motifs. The significance of the weak similarity with parts of the prokaryotic sigma factor, which performs an analogous function in bacteria, is uncertain^{1–4}.

This finding has interesting evolutionary, structural and functional implications. TFIID from organisms now extant may have arisen by duplication of a primordial monomer, followed by sequence divergence. But the duplication

of the same segment must be reflected in the structure of the protein, perhaps giving TFIID a partly symmetrical configuration. Such a symmetry could be relevant to the role of the protein in DNA recognition and binding; after all, the protein has to recognize a TATA sequence which is itself symmetrical (the same or a very similar nucleotide sequence on each of two opposing strands). It is also possible that the homologous domains bind similarly to two identical or different transcription factors.

Whatever their function, the amino-acid residues conserved in the homologous repeats must be expected to play an essential role in the proper functioning of TFIID.

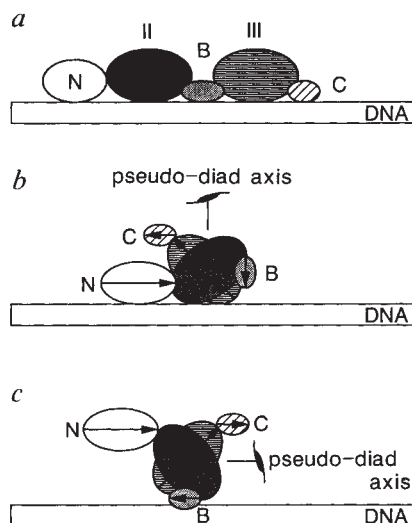
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SIR—The DNA sequence of yeast TFIID has recently been reported^{1,2}. By further sequence analysis I have revealed an interesting sequence repeat characteristic of a domain structure. The segment between amino-acid residues 67 and 131 shows striking sequence similarity to that between residues 157 and 222, which the authors of refs 1 and 2 failed to notice. Identical residues occur at 25 positions out of 66 (30 per cent), and if conservative amino-acid replacements such as Ala→Gly, Val→Leu, Asp→Glu are considered, the sequence similarity of these two domains is as high as 60 per cent. The architecture of TFIID is apparent from the presence of these duplicated domains within the protein. These domains have been discovered independently by Hoeijmakers (see above).

Of the residues of the N-terminal region (see figure) 65 per cent are hydrophilic, including Asp- or Glu 14 and Lys- or Arg 10. The B domain, which bridges domains II and III (see above for nomenclature), is rich in Lys and Arg residues. Horikoshi *et al.*¹ noted that part of this region might form an α -helix with Lys and Arg along one face. Weak sequence similarities between residues 171–212 of TFIID (within the proposed III domain) and the conserved 2.3 and 2.4 regions of bacterial σ -factors were noted^{1,2}. Horikoshi *et al.*¹ proposed that this region of TFIID might interact with the TATA element, as do bacterial σ factors⁴. The similarity to the 2.3 region is not convincing in the II domain, but both domains II and III show some similarity to the 2.4 region.

This domain structure immediately suggests ways in which TFIID might interact with DNA and other transcription factors. There are three symmetrically distinct ways to arrange domains II and III with respect to DNA. In model *a* (see figure), domains II and III are tandemly

placed with respect to the DNA molecule so that the basic region (B domain) is in contact with the TATA box. In models *b* and *c*, the protein chain is folded back at



Schematic models of the TFIID-DNA complex consistent with the five domain structure of TFIID: the N-terminal hydrophilic domain (N); the duplicated II and III domains; the B domain which bridges the II and III domains; and the C-terminal domain (C).

the B region so that II and III are related by a pseudo-diad axis. This axis of symmetry can have two distinct orientations relative to the DNA molecule. If domains II and III both interact with DNA, they should be structurally equivalent with

respect to DNA. Symmetry would then require the pseudo-diad axis to point into the minor or major groove (the TATA box has diad symmetry) as is the case for prokaryotic DNA-binding proteins such as λ , 434, Trp and Met repressors⁵. In this case the B domain cannot have close contact with DNA. In model *c*, the pseudo-diad axis is not involved in the interaction with DNA, the B domain binding directly to the TATA box. In this model domains II and III may stabilize the protein or bind to other factors.

The binding of TFIID to the TATA box promotes the binding of transcription factors such as TFIIA and TFIIB and the subsequent binding of RNA polymerase II (refs 6, 7). Yeast TFIID can replace mammalian TFIID to form active transcription-initiation complexes and it is interesting to see how the domain structure and amino-acid sequences have been conserved during evolution. Deletion or swapping of the II and III domains is an obvious experiment to determine their function in TFIID.

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1. Horikoshi, M. *et al.* *Nature* **341**, 299–303 (1989).
2. Hahn, S. *et al.* *Cell* **58**, 1173–1181 (1989).
3. Schmidt, M.C., Kao, C.C., Pei, R. & Berk, A. *Proc. natn. Acad. Sci. U.S.A.* **86**, 7785–7789 (1989).
4. Lillie, J.W. & Green, M.R. *Nature* **341**, 279–280 (1989).
5. Otwinowski, Z. *et al.* *Nature* **335**, 321–329 (1988).
6. Hahn, S. *et al.* *EMBO J.* **8**, 3379–3382 (1989).
7. Buratowski, S. *et al.* *Cell* **56**, 549–561 (1989).

Archaeobacterial or eocyte tree?

SIR—In response to my study¹, Gouy and Li² re-examined the tree of life and concluded that the archaeobacterial tree, rather than the eocyte tree, is supported. I would like to point out some flaws in their paper.

First, the authors assert that their preferred methods of phylogenetic analysis (augmented distance and maximum parsimony) are superior to evolutionary parsimony. Felsenstein, however, showed that maximum parsimony can artefactually select trees with juxtaposed long branches (as in the archaeobacterial tree) whenever taxa evolve at different rates³. Similarly, when rates vary at different positions by orders of magnitude (as in ribosomal RNA sequences), augmented distance can also select an archaeobacterial-like tree⁴. By contrast, evolutionary parsimony is the algorithm least sensitive to unequal rate effects^{5–7}. The mathematical assumptions of evolutionary parsimony are “mild without precedent”.

Although Gouy and Li report that “... the eocyte tree is strongly favoured...” by evolutionary parsimony analyses of a complete set of small subunit ribosomal RNAs, they use simulations with conspicuously unrealistic parameters

to discount this result. For example, in my original analysis of ribosomal RNA data, 1,400,000 nucleotide quartets (17,000 of these were independent) were analysed. Gouy and Li used just 900 quartets for their simulations. Of course, if only 900 quartets are simulated, evolutionary parsimony will support no tree. If a realistic number of quartets are used, evolutionary parsimony functions robustly and the strong support that it gives for the eocyte tree cannot be dismissed.

Likewise, simulations supporting Gouy and Li's favoured methods of reconstruction are erroneous. For example, they calculate branch lengths using augmented distances to estimate the number of substitutions. Shoemaker and Fitch⁸ showed that because of invariable sites, these lengths underestimate the actual distances by factors of 3 to 10. Because unequal-rate effects increase rapidly with sequence divergence, and branch lengths have been underestimated, Gouy and Li's simulations are unable to detect if algorithms fail.

Furthermore, Gouy and Li do not discuss why they assigned a non-zero length to the central branch of their eocyte model tree. Their reported χ^2 value for the eocyte

tree corresponds to a length for the central branch that is not significantly different from zero. But, they used a central branch length of 3.8 per cent in their simulations, longer even than that of their archaeobacterial model tree (1.5 per cent), thereby biasing the results towards their conclusions.

Gouy and Li argue that they have compensated for the faster evolution of large subunit sequences by visually selecting conservative positions to be analysed. But an independent augmented distance analysis³ of the "most conserved domains" at the 3' end of the large subunit sequence (48 sequences were used compared with the 9 used by Gouy and Li) supports the eocyte tree. Furthermore, Gouy and Li find (see below) that when the most slowly evolving eubacterial large subunit sequences are used (*Anacystis nidulans*, *Bacillus subtilis*, *Micrococcus luteus*), evolutionary parsimony supports the halobacterial tree (at the 5 per cent level) and not the archaeobacterial one. Clearly, analysis of the rapidly evolving large subunit sequences is subject to uncertainties of alignment and sequence selection.

It seems clear that Gouy and Li's large subunit analyses, confounded by high rates of evolution, are doubtful. On the other hand, Gouy and Li have confirmed the findings of my earlier analysis of small subunits¹, that is, that evolutionary parsimony supports the eocyte tree, and parsimony and distance matrix support the archaeobacterial one. Given their flawed simulations, and the large body of data supporting evolutionary parsimony as the preferred method of analysis, I favour the eocyte tree.

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GOUY AND LI REPLY—Two lines of evidence indicate that the evolutionary parsimony method is not suitable for analysing ribosomal RNA sequence data. First, the results obtained from different data sets are strongly incongruent. For the 22 small-subunit (SSU) and 22 large-subunit (LSU) ribosomal RNA sequences we used (only 9 species were presented² because of space limitation), the former strongly supported the eocyte tree whereas the latter strongly supported the archaeobacterial tree. Lake argues that LSU sequences are not suitable because they display, on the whole, a greater sequence divergence than SSU sequences. But for the regions we used, the rates in the LSU and SSU sequences are not very different (ref. 2, Fig. 1c). For the LSU data the χ^2 values for the archaeobacterial, the eocyte and the halobacterial trees are 34.4, 0.8 and 4.1, respectively. This leads to the question: if the eocyte tree is the

true tree, why have all the signals supporting this tree disappeared (the χ^2 value is only 0.8)?

Contrary to Lake's claim above that when the most slowly evolving eubacterial LSU sequences are used, the evolutionary parsimony method supports the eocyte tree, our application of this method to the same sequences with a different alignment supports the halobacterial tree. The evolutionary parsimony method, therefore, gives radically different results depending on the alignment used (Lake's alignment is probably not restricted to structurally conserved regions) and on the choice of species.

Lake¹ used 984 sites from each of 17 SSU sequences and obtained a probability of 2×10^{-6} for supporting the eocyte tree. It is doubtful that one can attain such a high resolution with such a limited amount of data. In our analysis² of 1,658 sites from only 8 LSU sequences, we obtained a probability of 5×10^{-6} for supporting the archaeobacterial tree. The two results^{1,2} are thus diametrically opposite to each other. For this reason we have serious doubts about the usefulness of the evolutionary parsimony method in dealing with the universal tree question, and in general we have reservations about the accuracy of the probability obtained from this method when more than four species are involved.

Lake also argues for the suitability of the SSU data, but our empirical test of the evolutionary parsimony method on the SSU sequences from humans, *Drosophila*, rice and *Physarum* erroneously grouped humans and rice in one clade. The reliability of this method for dealing with cases of deeper divergence is questionable.

By contrast, the neighbour-joining and maximum parsimony methods give completely congruent results in support of the archaeobacterial tree, whether the SSU and LSU data are used separately or jointly. The evolutionary parsimony method performs poorly probably because it assumes equal rates of transversal substitutions.

In our computer simulation², we used 900 nucleotides because that was the length of the SSU sequences used. Regardless of whether the archaeobacterial or the eocyte tree is used as a model both neighbour-joining and maximum parsimony methods are superior to evolution-

ary parsimony; as we noted², the branch lengths of the eocyte tree were estimated by Lake's method. The superiority of neighbour-joining to evolutionary parsimony was supported by a more extensive simulation¹⁰, including the case of large variation in rates among nucleotide sites.

The analysis by Bachellerie and Michot⁹ of partial sequences would be less reliable than our analysis of full length sequences. Finally, we note that the archaeobacterial tree is also supported by RNA polymerase sequence data¹¹.

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Blur into focus

SIR—Morgan and Benton¹ suggest that the human visual system does not have a general motion deblurring mechanism², because the threshold for discriminating the spacing between two bars is degraded significantly by image motion. However, image motion has only modest effects when compared with static spatial blur. For a pair of lines in motion, the interval discrimination threshold, ΔT_i , only doubles for an 8-fold change in image velocity (V), corresponding to $\Delta T_i \propto V^{0.3}$ over the range from 0.75 deg s⁻¹ to 6 deg s⁻¹. By contrast, Levi and Klein³ report that interval discrimination thresholds are directly proportional to static spatial blur (B) when the blur width is comparable to, or larger than, the spatial interval. Also, in a related psychophysical task, Watt and Morgan⁴ report that thresholds for discrimination of the extent of static spatial blur increase even more steeply ($\Delta T_b \propto B^{1.5}$). These observations suggest a marked difference in the effects of static blur versus motion blur, and they argue against the simple notion that discrimination thresholds are proportional to motion blur, which in turn is proportional to image velocity. Clearly, the issue deserves a more direct test, in which the effects of motion blur and static blur are compared on the same observers using otherwise identical stimuli and psychophysical tasks.

Without some form of deblurring process, the visual system would face major difficulties in accurately analysing moving images. For the high velocity condition (6 deg s⁻¹) in the Morgan and Benton task, the image traverses about 50 cones during the 100 ms estimated for the normal temporal integration period⁵. This introduces spatial blur and also reduces the intensity of the signal reaching each photoreceptor, thereby degrading its

1. Lake, J.A. *Nature* **331**, 184–186 (1988).
2. Gouy, M. & Li, W.-H. *Nature* **339**, 145–147 (1989).
3. Felsenstein, J. *Syst. Zool.* **27**, 401–410 (1979).
4. Lake, J.A. In *The Ribosome* (Am. Soc. Microbiol., in the press).
5. Holmquist, R., Miyamoto, M.M. & Goodman, M. *Molec. Biol. Evol.* **5**, 201–216 (1988).
6. Felsenstein, J. *Nature* **335**, 118 (1988).
7. Cavender, J. *Molec. Biol. Evol.* **6**, 301–316 (1989).
8. Shoemaker, J.S. & Fitch, W.M. *Molec. Biol. Evol.* **6**, 270–289 (1989).
9. Bachellerie, J.-P. & Michot, B. *Biochimie* **71**, 701 (1989).
10. Jin, L. & Nei, M. *Molec. Biol. Evol.* (in the press).
11. Pühler, G. et al. *Proc. natn. Acad. Sci. U.S.A.* **86**, 4569–4573 (1989).

signal-to-noise ratio. Nonetheless, at this speed the visual system can distinguish two intervals that differ spatially by less than 0.5 arc min (less than the spacing between cones), and temporally by only 1.4 ms. We contend that reliable extraction of detailed pattern information from moving images would be extremely difficult unless the visual system can make spatial comparisons that take into account the temporal delay at which different photoreceptors have been stimulated. This is the essence of any motion deblurring process^{2,6}.

Morgan and Benton confirm previous reports that Vernier acuity is only minimally degraded by image motion, and they interpret this by proposing that orientation-selective cells are exquisitely sensitive to the relative timing with which different parts of the receptive field are stimulated. This mechanism is tantamount to a form of motion deblurring as defined above. The need for it arises because Vernier acuity is more strongly impaired by static spatial blur^{4,7} than by motion blur.

Motion deblurring is but one example of visual processes that may involve dynamic transformations of the frame

of reference within which images are analysed⁶. Strategies for the dynamic control of information flow, such as that provided by putative neural 'shifter circuits' (ref. 8), may provide powerful means of overcoming the limits of fixed connectivity in biological systems, thereby improving the efficiency and accuracy of neural computations.

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1. Morgan, M. J. & Benton, S. *Nature* **340**, 385-386 (1989).
2. Burr, D. C., Ross, J. & Morrone, M. C. *Proc. R. Soc. B* **227**, 249-265 (1986).
3. Levi, D. M. & Klein, S. A. *Vision Res.* (submitted).
4. Watt, R. J. & Morgan, M. J. *Vision Res.* **23**, 1465-1477 (1983).
5. Burr, D. C. *Vision Res.* **19**, 835-837 (1979).
6. Van Essen, D. C. & Anderson, C. H. in *Computational Neuroscience* (ed. Schwartz, E. J.) 278-294 (MIT, Cambridge, in the press).
7. Williams, R. A., Enoch, J. M. & Essock, E. A. *Invest. Ophthalmol. Vis. Sci.* **25**, 389-399 (1984).
8. Anderson, C. H. & Van Essen, D. C. *Proc. natn. Acad. Sci. U.S.A.* **84**, 6297-6301 (1987).

Ecological power laws

SIR—Cohen¹ has commented that there is as yet no mechanistic explanation for the empirical ecological proportionalities observed by McNaughton *et al.*². The prospect of "linking previously known structural regularities in plant and animal ecology with new functional patterns" may be less distant than Cohen fears.

The simple power-law relationships reported by McNaughton *et al.* are between the biomass and activity of herbivores in a given ecosystem, and various measures of the annual productivity of the vegetation on which the herbivores graze. There is another example of an empirically observed proportionality in natural communities. This is the observation that in mature plant stands the mass of plant tissue per unit area varies as the $-3/2$ power of the number of plants per unit area^{3,4}. The relationship, known as the ' $-3/2$ power law', has attracted various attempts at a mechanistic explanation. Here I show that the data assembled by McNaughton *et al.* conform rather closely to one of these mechanistic explanations, the so-called core-skin hypothesis⁵. This postulates that plants function as a thin and energetically active skin over a massive and inactive core. The inactive core (xylem) is evidently 3-dimensional; the active skin (leaf, phloem and stem cortical tissues) is assumed to be 2-dimensional. The $-3/2$ power law follows directly from these assumptions⁵.

To a first approximation we may equate the annual production of 'skin' with McNaughton *et al.*'s net productivity of foliage (NFP); the annual production of 'core' with McNaughton *et al.*'s net primary productivity (NAP). McNaughton *et al.* report the following relationships between NFP, NAP and C (annual consumption of vegetation by herbivores):

$$\begin{aligned}\log C &= 1.38 (\log \text{NAP}) - 2.32 \\ \log C &= 2.04 (\log \text{NFP}) - 4.80\end{aligned}$$

The units are $\text{kJ m}^{-2} \text{yr}^{-1}$. Eliminating C, we have

$$\log \text{NFP} = 0.677 (\log \text{NAP}) + 1.22$$

Thus McNaughton *et al.*'s data suggest that NFP varies as the 0.677 power of NAP. The theoretical prediction from the core-skin hypothesis is 0.666. It would of course be better to estimate the relationship between NFP and NAP directly but the agreement between observation and prediction is striking.

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1. Cohen, J. E. *Nature* **341**, 104-105 (1989).
2. McNaughton, S. J., Oesterheld, M., Frank, D. A. & Williams, K. J. *Nature* **341**, 142-144 (1989).
3. Tadaki, Y. & Shidei, T. *Nippon Rin Gakkaishi* **41**, 341-349 (1959).
4. Westoby, M. *Adv. ecol. Res.* **14**, 167-225 (1984).
5. Hardwick, R. C. *Ann. Bot. Fenn.* **60**, 439-446 (1987).

Hiccups and human purpose

SIR—Hiccupping is a reflex consisting of spasmodic bursts of inspiratory activity followed within 35 milliseconds or so by abrupt glottic closure, so that the ventilatory effect is negligible¹. It is a universally experienced phenomenon², but is also pathologically associated with brainstem lesions^{2,3}, metabolic upsets² and some upper gastrointestinal disturbances². Hiccups have been contrasted with obviously useful reflexes (such as the cough and the sneeze) as being of no positive function^{1,2}. The hiccup has also been suggested as the vestige of a primitive reflex act from other primates, the significance of which has now been lost¹.

I have recently been conducting a small sample study of percutaneous fetal assessment ($n=1$). I have occasionally observed a regular repeated movement, apparently involving the trunk of the fetus. The subject's mother assures me that this is the subject hiccupping. Fetal hiccupping⁴ was reported in 1899 and confirmed after the development of ultrasound⁵ and may occur for up to 1.2% of intrauterine time⁶. This persists into neonatal life, when it has been calculated as occurring 3,000 times more often than in adults⁷. It has been suggested that fetal hiccups represent early respiratory movements in preparation for extrauterine life⁴.

I wish to propose that fetal hiccupping is a useful reflex to allow vigorous exercise of the respiratory inspiratory muscles without the inhalation of liquor. Immobilization of fetal muscle leads to disruption of development⁸ or atrophy, so that movement is required for normal development. Thus hiccupping, being the only opportunity for maximal inspiratory movement, is an essential and universal fetal reflex. The mechanism for the inhibition of this reflex in adults is not known, though one may speculate that it might be related to the changes in blood gases which alter the hiccup threshold experimentally¹.

The hiccup should be reclassified as an essential normal intrauterine reflex that may recur, like other primitive reflexes, in adult life.

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1. Newsome Davis, J. *Brain* **93**, 861-872 (1970).
2. Lewis, J. H. *J. clin. Gastroenterol.* **7**, 539-552 (1985).
3. Plum, F. & Posner, J. B. *The Diagnosis of Stupor and Coma* 3rd edn (Davis, Philadelphia, 1982).
4. Dunn, A. M. *Lancet* **ii**, 505 (1977).
5. Lewis, P. J. & Trudinger, B. *Lancet* **ii**, 355 (1977).
6. Woeden, E. E. *et al. Eur. J. Obstet. gynecol. Reprod. Biol.* **30**, 209-216 (1989).
7. Brouillette, R. T. *et al. J. Pediatrics* **96**, 219-255 (1980).
8. Reiser, P. J. *et al. Exp. Neurol.* **99**, 59-72 (1988).

Shadows of the past

J. L. Heilbron

German National Socialism and the Quest for Nuclear Power 1939–49. By Mark Walker. Cambridge University Press: 1989. Pp.290. £27.50, \$29.95.

IRRESPECTIVE of their political opinions, nuclear physicists alerted their governments to the possibilities of nuclear power and explosives immediately after the discovery of uranium fission. 'Uranium projects', supported by the state or the military, started up in Japan, the Soviet Union, the United States, Britain, France and Germany. We may not approve the actions of Walter Bothe, Otto Hahn, Paul Harteck, Werner Heisenberg and Carl Friedrich von Weizsäcker, to name only the most distinguished contributors to the German uranium project, but we should admit that, in working on it, they did not differ from their former colleagues in the Allied countries. Except in their success. As Heisenberg insinuated after the war, if guilt attached to physicists on either side, it belonged to the Allies, who had not only made a nuclear bomb, but used it.

Mark Walker does not indulge in moral judgements. He observes, however, that in working with or through or for the government or the military — and, what was also necessary from time to time, the SS — the German scientists could not help but be tainted. More than half of the 70 scientists or so who worked on the project were members of the Nazi party or of organizations close to it. Pursuit of the project entailed unsavoury activities: stealing Belgian and Czech uranium; commandeering Norsk Hydro to produce heavy water; grabbing machine tools from foreign countries; and, towards the end, when Allied bombing made the research centres in Berlin and Hamburg untenable, relocating laboratories with the help of slave labour. Still Walker does not judge. The scientists, he writes, remained true to their ideology, which placed the pursuit of scientific knowledge beyond politics. According to his interpretation of this hackneyed piece of special pleading, which can be traced back to the time of the French Revolution, it is apolitical — that is, professional — for a scientist to pursue basic research or offer objective advice whenever and by whomever requested. To do otherwise, to refuse work from antipathy to the politics of the requester, especially if the commission comes from constituted authority, would be political — that is, unprofessional and hence unscientific.

Walker does a good job in describing the circumstances in which the uranium projectors went about their apolitical research. Shortly after the first alert, German Army Ordnance took over

responsibility for most work on uranium fission and isotope separation and made the Kaiser-Wilhelm Institute for Physics in Berlin their main research site. No urgency was felt, because most responsible officials and the apolitical scientists

Better days — Heisenberg (second from left) discussing cosmic rays with other physicists at the University of Chicago in 1939.

supposed the war would not last long. Even so, the project made progress during the three years between the discovery of fission and the invasion of Russia. Its scientists tried various geometries of uranium, uranium oxide and hydrogenous moderators; concluded that, with enough natural uranium and heavy water, a self-sustaining 'uranium machine' could be constructed; investigated several promising ways of separating heavy water; and recognized that element 94, a necessary by-product of a uranium machine, would make a very powerful explosive. Only in plans for the separation of U-235 from natural uranium had the Germans fallen behind the Allies at the end of 1941.

As the military situation worsened, Army Ordnance made an internal study of the war potential of the uranium work it was supporting. Its experts dripped enthusiasm at the prospects of unanswerable all-powerful bombs and perfect power plants for submarines, and recommended scaling the project up to industrial dimensions. Their superiors, however, decided that the probability of producing a usable device remained too low and gave the uranium project to the Reich Research Council. That did not diminish support. The Army gave the project a gala farewell in February 1942, with a three-day

research conference followed by a series of semi-popular lectures by Harteck and Heisenberg *et al.* to military officers, government officials and representatives of industry. And, although everyone concerned with the project thereafter agreed that its goal was not weaponry, the Army and the Research Council confirmed its classification as *kriegswichtig*, important for the war effort.

In keeping with this classification, the project continued, under the general auspices of Reichsmarshal Herman Göring and Minister of Munitions Albert Speer. If anything, its urgency increased

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

as its prospects declined. The sabotage of Norsk Hydro, the failure to find an effective way to separate uranium isotopes, the loss of essential engineering facilities at I.G. Farben, the need to dig underground laboratories and then to flee altogether, only intensified the drive to make an operational uranium machine. The reasons are not hard to find. For one, the German scientists wanted an accomplishment to show the world that even after the Nazis had all but eliminated modern physics from the universities and the Allies had all but obliterated physics research laboratories, German nuclear science could still stand high. (The shortage of physicists was so great in 1940 that, according to Walker, industries would fight to hire physics students who had flunked school.) For another, the German scientists believed that they did stand high — that they had outdistanced the Allies in work towards a uranium machine. The further they could get by the war's end, the higher the value the victors would place on their apolitical advice.

The US invasion forces rounded up the scientific leaders of the German uranium project and sent them to Britain for interrogation. The Germans could scarcely believe the news about Hiroshima. They recorded recriminations, as reported by

Daniel Irving in the *Virus House* (the pioneering study of Walker's subject), made clear how great a blow to their self-image was the discovery that the Allies had accomplished what they had not come close to achieving. Hahn accused the others of being second rate; they had no stock in trade; they had not done outstanding science; they had suffered a defeat as inglorious as the Luftwaffe's. Immediately on recovery from the shock of Hiroshima, the scientists began to fashion what Walker calls "the myth of the German atomic bomb". Heisenberg was the leading mythologist. In a stream of apologia, he claimed that the German project had been conducted as pure science, that the scientists had been in charge all along, that they would never have made a bomb for Hitler, that only a very few scientists were "political" — that is, worked to secure the goals of the Nazi regime — and that the leaders of the project conducted their research according to the norms of apolitical science.

The apologia irritated scientists in the Allied countries, especially emigrés, but it worked well enough with military government that Heisenberg became a respected character witness on behalf of scientists undergoing denazification. He even put in a good word for Johannes Stark, one of the fountainheads of *Deutsche Physik*, an anti-Semitic, anti-modern movement directed against Einstein and "white Jews", like Heisenberg, who taught theoretical physics in the Jewish style. Walker makes the penetrating observation that for Heisenberg and the other nuclear scientists, it was not necessary to brand their former enemies in *Deutsche Physik* as Nazis, only as poor physicists who had used the apparatus of the state to obtain positions they did not deserve. That allowed the useful line-up of the competent and apolitical against the incompetent and political.

Walker permits his protagonists to hide behind his conception of their conception of professional scientific behaviour. He gives the case of Harteck, who tried to persuade Norwegian scientists to co-operate in increasing the supply of heavy water, which, he said, would be used only for basic research. Walker finds "no reason to doubt his sincerity", but allows that the minefields, barbed wire and SS men surrounding Norsk Hydro might have made the Norwegians question it. Then there is Heisenberg, travelling up and down the occupied territories as an ambassador of German culture, justifying the ruthlessness of German administration as the lesser of two evils, the other being democracy. Later Heisenberg claimed that his behaviour amounted to active opposition to the regime. This is almost too much for Walker. "[It] is very difficult to reconcile with his actions." Yet Walker writes that in making his claim

Heisenberg was "sincere." Again, Heisenberg tried to give the impression that the German project did not proceed as quickly as it could have because he and his circle held back for moral reasons. Walker says that "Heisenberg's article should be taken as what it was, an honest sincere account of his retrospective misgivings. . . . However, this article presented an inaccurate account of [his] actions." Double-think is contagious.

On the apolitical or professional level, Walker has constructed a very good book. He has studied captured documents, gov-

ernment and military reports, and archival material, and integrated the results with interviews he has conducted with veterans of the uranium project. The integration, which presents the story of the project against the changing fortunes of the war, is easy to follow. It provides the facts in sufficient detail and context to permit readers to construct their own solutions to whatever moral or ethical questions they may think it raises. □

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Fact and fantasy

Jeffrey A. McNeely

Tom Slick and the Search for the Yeti. By Loren Coleman. *Faber and Faber*: 1989. Pp. 176. Pbk \$11.95.

As human influences reach further into the world's shrinking wildernesses, people continue to be fascinated by the possible existence of unknown animals. Each Himalayan climbing season brings new

which is attributed in this book to his step-father's kidnapping in the 1930s by the infamous American gangster "Machine Gun" Kelly. Slick's death in a mysterious aeroplane crash (some claiming a link to power struggles over the \$140-million budget of the Southwest Research Institute) at the age of 46 in 1962 spelled the end of significant funding for cryptozoology.

Although Slick was sympathetic to the idea of unknown animals, he also sought proof before claiming that such creatures actually existed. Loren Coleman has no

Fortean Picture Library

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The American Big Foot — who needs further proof?

sightings of tracks or other evidence of the Yeti, the famous man-like creature said to haunt the snowy peaks along the Nepal-Tibet border.

One of the strongest supporters of a systematic approach to cryptozoology, the study of animals which have not yet been proved to exist, was Tom Slick. No fly-by-night publicity seeker, Slick made millions from oil and real-estate and was the founder of the Southwest Research Institute, one of the largest nonprofit applied research institutes in the world (behind only the Batelle Memorial Institute and the Stanford Research Institute). Despite the inherent public appeal of cryptozoology, Slick avoided publicity,

such concerns, stating unequivocally that Yetis do exist but not backing up this assertion with any new evidence. His short book contains 134 pages, 20 of them directly reprinted from one of the classics of cryptozoology, *On the Track of Unknown Animals*, by Bernard Heuvelmans (Hart-Davis, London, 1958). The information provided by several expeditions into northeastern Nepal in the late 1950s reveals more about the searchers than the Yeti. Members of the 1959 Slick expedition stole the thumb and phalanx of a mummified hand held at the lamasery of Pangboche, which was claimed by the Sherpas to be a hand of a Yeti. Slick's minions substituted the thumb and phalanx from a human skeleton, thereby throwing into confusion all subsequent investigation of the Pangboche hand. The relics were taken to India, where they were smuggled to London by none other than the film actor James Stewart, an old friend of Slick. Although the insights into Tom Slick and his cryptozoology expeditions are intriguing, they shed no new light on the Yeti itself, which continues to occupy the remote wildernesses of the human spirit. □

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Moving times

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Molecular Genetics in Diseases of Brain, Nerve and Muscle. Edited by Lewis P. Rowland, Donald S. Wood, Eric A. Schon and Salvatore DiMauro. *Oxford University Press: 1989. Pp.481. £45.*

WHAT a peculiar and exhilarating time it is to be working in human genetics! The surprising results of today are with us for the briefest time, only to be replaced by the amazing results of tomorrow and the quite unbelievable results of the day after tomorrow. To paraphrase an old aphorism, the utterly impossible, we must all now be convinced, will take just a bit longer. The speed with which genetic science, clinical application and the art and ethics of medicine are becoming so intricately intertwined is dazzling. The distinctions between these previously disparate disciplines, once so clear, are melting disquietingly and uncontrollably away, making it increasingly difficult for any of us to take refuge in our parochial past.

The multi-authored *Molecular Genetics in Diseases of Brain, Nerve and Muscle*, edited by Rowland, Wood, Schon and DiMauro, well illustrates some of the problems faced by those who would try to use the format of a single volume — in this case an expanded symposium report — to describe such an explosive field to a clinical audience and to examine the impact of molecular genetics on clinical medicine, in particular on neuromuscular and neurological diseases. All the appropriate and *de rigueur* topics, at least as they were recognized two years ago, are conveniently present and most are covered reasonably well. Most of the flavour of the current revolution in medical genetics is in evidence, and a strength of this volume lies in its enumeration of some of the fields in which major upheavals in the approach to neuromuscular disease have already occurred. The book's principal weakness lies in its transience. For whom will it be of more than casual interest one or two short years from now?

There are the usual trivial clashes in style, redundancies and variations in the quality of writing, ranging from eloquent (Wexler's chapter, "The Oracle of DNA") to the more ordinary to the slightly tortured. Discussions of the basics of gene structure, genome organization and genetic analysis are generally clear and appropriately aimed at an interested clinical audience, although most of this material is more thoroughly described and illustrated in several other sources. Especially good are the descriptions of library screening and sequencing (Monaco), construction of physical maps (Smith and Cantor) and two chapters on

linkage and genetic analysis. Some discussions are a bit skimpy and will not be easily understood by clinicians (many may wonder to what the adjective "restriction" in restriction enzymes refers — they're certainly never told). Amazingly, there is even a chapter describing mendelian and non-mendelian inheritance without the use of a single pedigree. Most of the illustrations are of good quality and make their points, although figures adapted from other sources or taken from slides are occasionally almost incomprehensible.

The most satisfying and useful parts of the book for clinicians are the disease-specific chapters, including excellent discussions of fragile X syndrome (Davies), neurogenetic diseases (Gusella), Duchenne muscular dystrophy (Kunkel) and the many other fascinating clinical targets for genetic analysis. To the clinical audience, this volume therefore represents a handy, reasonably complete and comprehensible, albeit a fleeting, view of a burgeoning field. □

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Fossil forays

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The Human Revolution: Behavioural and Biological Perspectives on the Origins of Modern Humans. Edited by Paul Mellars and Chris Stringer. *Edinburgh University Press: 1989. Pp.800. £65. Distributed by Princeton University Press in the United States, \$65.*

IN 1986, the World Archaeological Congress was held at Southampton in the United Kingdom despite the absence of participants from South Africa which had earlier threatened to bring about its cancellation. The price paid was that one or two symposia planned to be held at Southampton were withdrawn. The meeting at Cambridge held in the spring of 1987 giving rise to this book was one of these. The Cambridge meeting was not without its political problems, as the editors readily concede in a preface that sets out the circumstances of the decision to withdraw from the World Archaeological Congress. Despite the eminence of those who did take part, there are notable omissions from the list of contributors.

In the event, the symposium was clearly a success and this volume is the first of two planned to document the meeting. The largest section contains papers on human palaeontology and evolution; the other two sections contain chapters on more general conclusions from the behavioural and archaeological data presented at the

meeting. The companion volume promises to deal with more specialized case studies.

The problem addressed at the meeting is that of the origin of our own species, *Homo sapiens*, as opposed to other hominid creatures, such as ape-men from Africa or *H. erectus* from the Far East or wherever. That this is a problem will perhaps come as a surprise to the non-specialist as it is broadly true that the further back in time that is sought, the less the palaeoanthropological evidence and the greater the controversy generated by published conclusions. The focus of attention on our own species in recent years seems to rest on three factors: the discovery of new fossils and the reinterpretation of others; improvements in established dating methods and the discovery of new ones; and innovations in statistical analysis, microscope analysis and in taxonomic theory. The outdated but still persistent correlation of anatomical type with tool type (for example, Upper Palaeolithic tools equals modern *H. sapiens*) has been challenged by new data from Skhül and Qafzeh (Middle Palaeolithic tools and modern skeletal form) as well as Saint Césaire (Neanderthal partial skeleton and an Upper Palaeolithic industry). These challenges to conventional thinking are tending to decouple anatomical form from behaviour, at least in terms of evidence from stone tools.

One of the main debates centres on the question of the primacy of Africa as the centre of the evolution of anatomically and genetically modern *H. sapiens* (the so-called "Noah's Ark" hypothesis) as opposed to a multiregional theory of sapient evolution that sees modern man evolving as a polytypic species from more than one centre. The "Out of Africa" hypothesis has received a good deal of support in recent years from new dating evidence for specimens from sites such as Klasies River Mouth and Border Cave. This evidence seems to point to modern human anatomy originating between 70,000 and 100,000 years before present and in some cases, such as Omo, even earlier. In addition, new evidence derived from DNA and other molecular-genetics studies seems to indicate unequivocally that the modern human genotype arose in Africa.

This view of sapient evolution is attacked forcefully by Wolpoff, both in terms of the unreliability of the dating of the so-called anatomically modern human remains from African sites and the evidence relied upon by molecular geneticists for the calibration of the mutation rates of mitochondrial DNA. A slower mutation rate could retard the dispersal of populations from Africa to about 850,000 years before present, a date that corresponds to the widely accepted dispersal of *H. erectus*

out of Africa in the early Pleistocene.

Consideration of the evidence from the Middle East reveals equally interesting debates, including strong support for the earlier date of 90,000–100,000 years before present for the origin of the modern forms at Qafzeh and a younger date (50,000–60,000 years before present) for the very robust Neanderthal skeleton at Kebara. A simple linear evolution from the Neanderthals of Tabūn and Shanidar to the anatomically modern forms of Skhūl and Qafzeh now seems unlikely.

The authors of the African archaeological papers consider the transition from the middle to the later Palaeolithic and suggest that more complex behavioural patterns than previously thought can be inferred. In Europe, most of the occurrences of anatomically modern man seem to coincide with 'Aurignacian' industries, but other industrial variants are more mixed. The authors of several other

papers consider the position in the Far East and Australasia as well as inferences concerning mental abilities, language and art, all of which raise fascinating questions fundamental to our appreciation of the evolution of modern man.

There is little doubt that this is an important book for all palaeoanthropologists and archaeologists — it is international in its scope and broad in its treatment of the exciting issues central to modern thinking in these fields. Although it is sad that it cannot take its place with the other volumes that emerged from the World Archaeological Congress, for which it was so painstakingly planned, it is a credit to its contributors and its editors as a most valuable contribution to the literature of human evolutionary studies. □

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Out of the bag

Stuart Sutherland

Mind, Brain and the Quantum. By Michael Lockwood. Basil Blackwell: 1989. Pp. 365. £25, \$29.95.

RUMINATING on the relationships between the mind, the brain and quantum mechanics is not merely fashionable, it is becoming an industry. Philosophers apply quantum theory to the problems of consciousness, while physicists use consciousness to solve the paradoxes of quantum theory. Michael Lockwood does both.

Lockwood begins by dismissing the doctrine of functionalism, the idea that conscious states are to be thought of as brain states classified in terms of their causal relations with one another and with the external world. In doing so, he uses ingenious variants of the arguments from qualia, which basically depend on the fact that one could conceive of entities having states with the same functional relationships as those of mental states, but lacking conscious experience or having conscious experiences totally different from our own. He adopts a form of the identity theory — which is that consciousness is the same thing under another guise as certain brain states.

Mind, Brain and the Quantum is nothing if not bold. Its first daring stroke is an attempt to demonstrate that consciousness must be spatially located. Lockwood argues that because on the special theory of relatively "any two events which are temporally separated with respect to one frame of reference must be spatially separated with respect to some other frame" and because mental events are

located in time, they must be located in space. Now consider a physical event A that causes a mental state M which in turn causes another physical event C: the mental event must lie within the future light cone of A and the past light cone of C. According to Lockwood, this places it in some part of the brain, but the argument contains a *petitio principii*, as it rests on the assumption that consciousness is in some sense a physical entity.

In resolving the problem of Schrödinger's cat, Lockwood starts from H. Everett's theory that when a quantum system influences the macroscopic world, every possible outcome of the system occurs: the world branches into an infinity of different worlds. In contrast to Everett, Lockwood argues that macroscopic events can be in different and incompatible states simultaneously (Schrödinger's cat is both dead and alive) until they are observed, at which point each state enters one of a parallel stream of minds all belonging to the same observer. Although the observer is conscious of only one, he has an infinity of different lines of consciousness (biographies), each representing the sequence of macroscopic events produced by different outcomes of quantal events. In other words, Lockwood has changed Everett's notion that splitting into different worlds occurs whenever an unobserved quantum event influences the macroscopic world into the theory that the splitting takes place in consciousness (on observation). I am uncertain just what this gloss on Everett buys. According to Lockwood, the biography we happen to be in is related to the past by memory, but what happens when memories are false is not clear. Nor, come to that, is the status of Schrödinger's cat. Because we can always be mistaken, one can ask what it would

mean to perceive the cat as dead, when it was really alive. It would be as embarrassing for Lockwood's theory as for the observer, if the wretched animal subsequently turned up demanding a bowl of milk.

Many of Lockwood's speculations about the brain are even more extraordinary. He suggests that there are phenomenological events of which we are not aware. If we are presented with monochromatic light of three wavelengths close together in the spectrum, we may be able to distinguish the lowest from the highest while not being able to tell the middle one from either of the other two. He concludes that the lowest and the highest must both be phenomenologically distinct from the middle one, even though we are not conscious of the distinction. But surely all one can conclude is that the members of the neighbouring pairs of wavelengths produce a difference in the activity of the nervous system that is too small to register in consciousness.

A further novel argument is that because conscious states are continuous whereas the firing of neurons is discrete, nerve impulses cannot underlie consciousness. This leads to the revolutionary suggestion that consciousness resides in the polarization of the dendrites, which Lockwood alleges is continuous. The argument can be refuted by a *reductio ad absurdum*. The polarization of neurons is itself no more discrete than their firing rates, for it depends on the number of individual ions lying on either side of the membrane.

Perhaps Lockwood's wildest fling is to suggest that the brain is a quantum computer. He struggles to overcome the problem that such a computer could only function in extreme cold, asserting that it might work if the temperature were kept constant — but the ratiocinatory abilities of alligators are far from negligible.

The book is densely but well written and deserves to be widely read. I have not been able to do justice to its curious mixture of careful and often highly ingenious philosophical arguments and its bizarre but interesting speculations. Lockwood, with characteristic wit, writes "there is nothing so obvious that a philosopher cannot be found to deny it". Philosophers may be hard put to find anything obvious in this book, but it is none the worse for that. □

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■ Also recently published by Basil Blackwell, *In and Out of the Black Box: On the Philosophy of Cognition* by D. W. Hamlyn is an account of mental life and its effects on behaviour, meaning and consciousness. Price £25, \$29.95.

■ *From Reading to Neurons*, edited by A. M. Galaburda, is a formal approach to developmental disorders of cognition. Published by MIT Press, price \$45, £40.50.

Regulation of the mevalonate pathway

Joseph L. Goldstein & Michael S. Brown

The mevalonate pathway produces isoprenoids that are vital for diverse cellular functions, ranging from cholesterol synthesis to growth control. Several mechanisms for feedback regulation of low-density-lipoprotein receptors and of two enzymes involved in mevalonate biosynthesis ensure the production of sufficient mevalonate for several end-products. Manipulation of this regulatory system could be useful in treating certain forms of cancer as well as heart disease.

A FINELY tuned mechanism regulates the biosynthesis of mevalonate, the precursor of isoprenoid groups that are incorporated into more than a dozen classes of end-products (Fig. 1). These include: sterols, especially cholesterol, involved in membrane structure; haem A and ubiquinone, which partake in electron transport; dolichol, required for glycoprotein synthesis; isopentenyladenine, present in some transfer RNAs; and intercellular messengers, such as cytokines in plants, farnesylated mating factors in fungi, juvenile hormones in insects, and steroid hormones in animals. Interest in the regulatory importance of mevalonate was heightened recently by the discovery that growth-regulating p21^{ras} proteins¹⁻³, encoded by *ras* proto-oncogenes and oncogenes, and nuclear envelope proteins⁴⁻⁶, are covalently attached to farnesyl residues, which anchor them to cell membranes. Inhibition of mevalonate synthesis prevents farnesylation of these proteins¹⁻⁶ and blocks cell growth⁷.

To ensure a constant production of the multiple isoprenoid compounds at all stages of growth, cells must precisely regulate mevalonate synthesis while avoiding overaccumulation of potentially toxic products such as cholesterol⁷. In the past few years the crucial genes that control mevalonate synthesis have been cloned, and the molecular mechanisms for mevalonate regulation are beginning to be unravelled. We review recent progress in this area and discuss the relation between mevalonate synthesis, cholesterol homeostasis and cell proliferation.

Balancing external and internal cholesterol

Cells from higher animals face a complex problem in regulating mevalonate synthesis because cholesterol, the bulk end-product of mevalonate metabolism, is derived from plasma low-density lipoprotein (LDL), which enters the cell by receptor-mediated endocytosis, as well as from synthesis within the cell (Fig. 1). Each cell must balance these external and internal sources so as to sustain mevalonate synthesis while avoiding sterol overaccumulation. This balance is achieved through feedback regulation of at least two sequential enzymes in mevalonate synthesis, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) synthase and HMG-CoA reductase, and also of LDL receptors (Fig. 1). In the absence of LDL, animal cells maintain high activities of the two enzymes, thereby synthesizing mevalonate for production of cholesterol as well as the nonsterol products. When LDL is present, HMG-CoA synthase and reductase activities decline by more than 90%, and the cells produce only the small amounts of mevalonate needed for the nonsterol end-products⁷. If excess mevalonate is supplied externally together with LDL, the residual activity of HMG-CoA reductase is abolished and mevalonate production is terminated. When cellular sterols rise or when cell growth ceases and cholesterol demand declines, the LDL receptor gene is repressed, further averting cholesterol overaccumulation⁸.

In addition to regulating mevalonate synthesis, cells regulate mevalonate disposition. The enzymes of the non-sterol pathways generally have higher affinities than those of the sterol pathway for mevalonate-derived substrates⁷. So, when mevalonate is limiting, it is preferentially shunted into the high-affinity non-sterol pathways. After prolonged incubation of cells with sterols,

squalene synthetase, the first committed enzyme of sterol synthesis, is suppressed, further limiting the incorporation of mevalonate into sterols⁷. Other enzymes of mevalonate metabolism, including acetoacetyl-CoA thiolase⁹ and prenyltransferase¹⁰, are also regulated by sterols, but the quantitative role of these regulatory steps has not yet been established.

Sterol-mediated regulation of transcription

The cloning from animal cells in recent years of the genes for the LDL receptor¹¹, HMG-CoA synthase¹² and HMG-CoA reductase¹³ has made it possible to investigate the mechanisms by which these gene products are regulated by sterols. One mechanism is transcriptional regulation. Figure 2 illustrates the coordinate induction and repression of the messenger RNAs for the LDL receptor and HMG-CoA synthase and reductase in animal cells that were cultured in the absence and presence of sterols.

To study in detail how transcriptional regulation is achieved, we focused our attention on the 5' flanking regions of the genes,

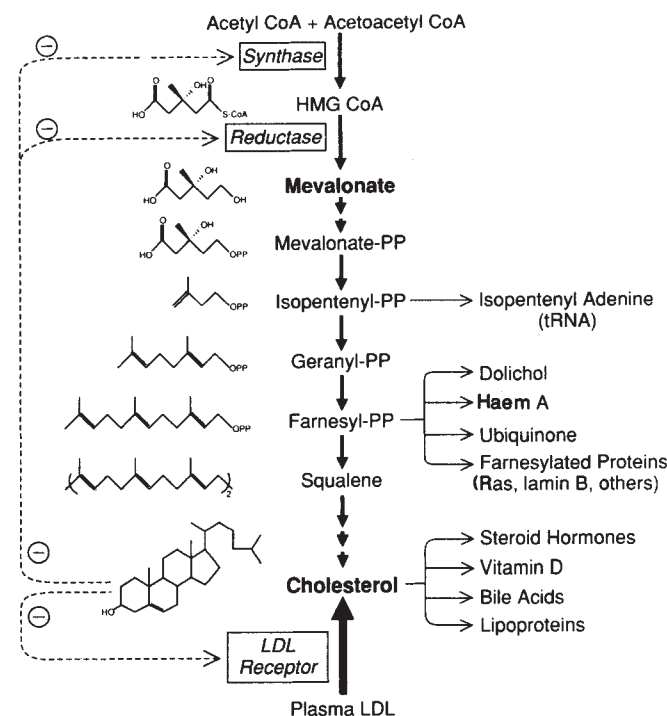


FIG. 1 The mevalonate pathway in animal cells. The bulk product of mevalonate metabolism, cholesterol, is obtained from two sources: (1) endogenously, by synthesis from acetyl-CoA through mevalonate; and (2) exogenously, from receptor-mediated uptake of plasma LDL. Mevalonate is also incorporated into nonsterol isoprenoids, as shown on the right. Mevalonate homeostasis is achieved through: (1) sterol-mediated feedback repression of the genes for HMG-CoA synthase, HMG-CoA reductase and the LDL receptor, as shown on the left; and (2) post-transcriptional regulation of HMG-CoA reductase by one of the nonsterol isoprenoids shown on the right.

through which sterols were expected to exert their effects. As anticipated, when Chinese hamster ovary (CHO) cells were transfected with fusion genes in which the 5' flanking regions of any one of the three genes were placed upstream of a 'reporter' gene, the fusion genes were transcribed in the absence of sterols and were repressed when sterols accumulated¹⁴⁻²⁰. Linker-scanner mutations in the 5' flanking regions of all three genes traced the principal regulatory activity to a short segment that contained a closely related sequence, which has been designated sterol regulatory element-1 (SRE-1). Like enhancers, SRE-1 sequences can function in either orientation. The SRE-1 is also capable of conferring sterol regulation when it is incorporated into the promoter for the herpes simplex virus gene for thymidine kinase^{15,17-19}. Figure 3 shows the consensus sequence of SRE-1 and the locations of this element in the promoter regions of the three regulated genes.

In the LDL receptor gene, the SRE-1 is a conditional positive element that enhances transcription in the absence of sterols but not when they are present²⁰. The SRE-1 synergizes with two nearby sequences that are relatively weak binding sites for the general transcription factor Sp1 (ref. 17). The two Sp1-binding sites and the SRE-1 are each located in 16-base pair (bp) sequences that are imperfect repeats of each other¹⁵⁻¹⁷ (Fig. 3c). *In vitro*-purified Sp1 stimulates transcription driven by the promoter of the LDL receptor gene (T. F. Osborne *et al.*, unpublished observations). *In vivo*, the SRE-1 and the two Sp1 sequences are all necessary for high-level transcription in the absence of sterols¹⁶. Point mutations in the SRE-1 prevent the induction of transcription that would otherwise occur when cells are deprived of sterols, but they do not affect the basal level of transcription that occurs in the presence of sterols^{17,20}. Evidence¹⁵⁻¹⁷ indicates that repeat 3 is a relatively weak binding site for Sp1 and that high-level transcription requires reinforcement from an enhancing protein that binds to the SRE-1 in repeat 2. Inactivation of this enhancing protein in the presence of sterols would account for the sterol-mediated decline in transcription of the LDL receptor²⁰.

The two SRE-1 sequences in the promoter of the HMG-CoA synthase gene (Fig. 3a) behave similarly to the single SRE-1 in the promoter of the receptor gene. Point mutations in either of them limit the induction of transcription in sterol-deprived cells, but do not affect transcription in the presence of sterols¹⁹. The synthase promoter also contains another short stretch of DNA,

designated SRE-2, whose disruption causes a partial loss of inducibility. When all three elements in the synthase promoter are mutated, transcription in the presence of sterols is unaffected, but there is no longer any induction when sterols are removed (ref. 19; J. R. Smith *et al.*, unpublished observations). All three SREs therefore act in a conditionally positive fashion, as in the promoter of the LDL receptor gene.

The role of the single SRE-1 in the promoter of the HMG-CoA reductase gene (Fig. 3b) is not as well established as in the other two promoters; but there is a suggestion that, unlike in the other two promoters, it actively represses transcription in the presence of sterols. The evidence comes from a 10-bp scramble mutation that altered six out of eight nucleotides in the SRE-1 and produced in transfected cells constitutively high transcription in the presence or absence of sterols¹⁸. These results cannot be interpreted unequivocally without point mutations, because the 10-bp scramble mutation could have affected the response to other factors in addition to the putative protein that recognizes the SRE-1. This is quite possible because the SRE-1 in the reductase promoter is located in the midst of a cluster of eight protein-binding sites, six of which bind proteins belonging to a family of transcription factors designated the CTF and NF-I group (NF-I, nuclear factor-1; CTF, CCAAT-binding transcription factors; refs 21-23).

Rajavashisth *et al.*²⁴ have recently cloned a cDNA encoding a 19K protein with seven zinc-finger repeats that binds to a single-stranded oligonucleotide corresponding to the sequence of SRE-1. Intensive studies are now under way to determine whether this protein is a physiological regulator of SRE-1.

Post-transcriptional control of HMG CoA reductase

HMG-CoA reductase is among the most highly regulated enzymes in nature. The complexity of this regulation was first revealed through the use of potent reductase inhibitors from fungi. These agents, which include compactin and lovastatin (mevinolin), competitively inhibit the enzyme at concentrations of less than 10^{-8} M (ref. 25). In cultured cells, they block the synthesis of mevalonate and trigger adaptive reactions that yield a 200-fold increase in reductase protein within a few hours^{26,27}. This induced protein is inactive in cells because it is blocked by the inhibitor, but it can be detected by enzyme assays *in vitro* after the inhibitor is removed by dilution or dialysis. The 200-fold increase in reductase protein is the multiplicative effect of smaller changes occurring at three levels (Table 1): induction of transcription produces an eightfold increase in mRNA levels, each mRNA is translated at a fivefold higher rate, and the enzyme molecules are degraded fivefold more slowly²⁷.

The 200-fold increase in enzyme can be reversed within hours by incubating compactin-treated cells with large amounts of mevalonate (10 mM) or with smaller amounts of mevalonate (<1 mM) together with plasma LDL^{7,26,27}. LDL alone suppresses the enzyme by only eightfold. These findings led to the concept⁷ that HMG-CoA reductase is controlled by several feedback-regulation mechanisms. Physiologically, full reduction in enzyme activity requires both a sterol that can be derived from LDL and a non-sterol metabolite that must be synthesized from mevalonate.

Recent evidence indicates that the sterol and non-sterol metabolites act at different levels. As described above, sterols repress transcription through effects on the SRE-1. But even when sterols are saturating, reductase mRNA continues to be transcribed at about one-eighth of the maximal rate²⁷. The rate of translation of this mRNA is dictated by the cell's demand for non-sterol isoprenoids. When mevalonate production is blocked by a reductase inhibitor, the mRNA is efficiently translated even in the presence of sterols, but when the non-sterol requirements are satisfied by administration of exogenous mevalonate together with sterols, translation of reductase mRNA is reduced by fivefold²⁷. Similar results are obtained without reductase inhibitors in a mutant line of CHO cells with a defect in HMG-CoA

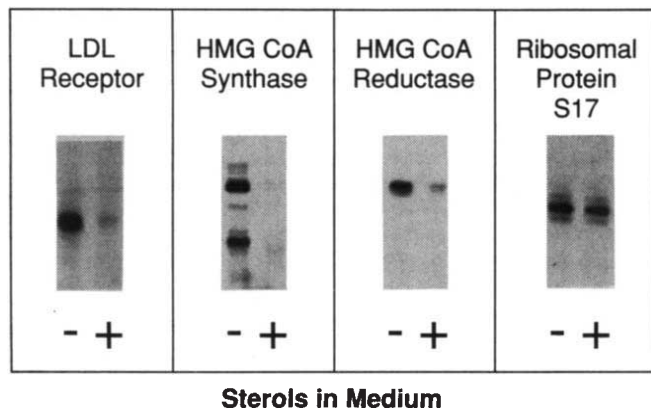


FIG. 2 Coordinate regulation of mRNA for the LDL receptor, HMG-CoA synthase and HMG-CoA reductase in CHO cells. Cells were incubated for 24 h in the absence (-) or presence (+) of a mixture of $0.6 \mu\text{g ml}^{-1}$ 25-hydroxycholesterol with $10 \mu\text{g ml}^{-1}$ cholesterol. Total RNA was isolated, and an aliquot was subjected to quantitative S1 nuclease analysis (for LDL receptor and reductase mRNA) or primer extension analysis (for synthase and ribosomal protein S17 mRNA) (see ref. 61 for details). The gels were exposed to X-ray film for 12-47 h at -70°C . The synthase gene produces two transcripts owing to alternative splicing of an intron in the 5' untranslated region⁸⁴.

TABLE 1 Mevalonate-mediated regulation of HMG-CoA reductase

Level of control	Mevalonate-derived regulatory agent		Proposed target for regulation	Amplitude of change
	Sterol	Nonsterol		
Transcriptional	+	—	SRE-1 sequence in promoter	8-fold
Post-transcriptional				
Translation of mRNA	—	+	Complex 5' untranslated region	5-fold
Degradation of protein	+	+	Seven membrane-spanning regions in N-terminal domain	5-fold
Inactivation of enzyme	—	—	Serine and threonine residues in C-terminal catalytic domain phosphorylated by AMP-dependent kinase	?

synthase that blocks synthesis of mevalonate²⁸.

Insight into the mechanisms that achieve post-transcriptional control of reductase, not only at the level of translation but also through degradation of the enzyme, have emerged from studies of a remarkable line of cultured CHO cells, designated UT-1 cells²⁹, which are selected for growth in high levels of compactin. These compactin-resistant cells produce enormous amounts of reductase (1% of total cell protein), owing to a 15-fold amplification of the reductase gene, an increased rate of transcription, and a decreased rate of protein degradation³⁰. (The high levels of reductase mRNA in UT-1 cells allowed the initial cloning of the complementary DNA through differential hybridization even before the intact enzyme had been purified³¹.)

Analysis of reductase in UT-1 cells produced two unexpected findings that could relate to post-transcriptional regulation. First, the reductase gene lacks the TATA-sequence box that determines the transcription initiation site in most genes, and it initiates transcription from multiple sites¹³, a pattern that was subsequently found in other 'housekeeping' genes³². Each of the initiation sites uses a different splice donor site for an intron in the 5' untranslated region³³, thus producing mRNAs with 5' untranslated regions that range from 68 to 670 nucleotides in length either with or without up to eight AUG codons upstream of the initiator methionine.

It is possible but not yet proven that the differential splicing produces one class of mRNAs whose translation is regulated by a mevalonate-derived substance. Mevalonate gives rise to isopentyladenine, which is located adjacent to the anticodon loops in certain tRNAs³⁴ and which could regulate translation of reductase. Alternatively, this regulation could be mediated by a farnesylated protein (see below). It is noteworthy that the

5' untranslated region of HMG-CoA synthase also undergoes differential splicing¹², but there is no information as to whether it is translationally controlled.

The second unexpected finding from the hamster UT-1 cell experiments is that HMG-CoA reductase is a structurally complex protein whose degradation rate is governed by an unusual hydrophobic domain³⁵. The hamster enzyme is a glycoprotein of 887 amino acids³¹ which is bound to the membranes of the nuclear envelope and smooth endoplasmic reticulum (ER)^{29,36}. All of the catalytic activity is contained in the C-terminal 548 amino acids, which project into the cytosol. The N-terminal domain contains alternating stretches of hydrophilic and hydrophobic amino acids that are predicted to traverse the membrane seven times³⁵. A similar structure is found in the enzyme from all eukaryotic species examined³⁷⁻⁴², except *Arabidopsis*, which contains only one membrane-spanning region⁴³. Several membrane spans are unusual for a protein of the ER. This organization resembles that of ion channels or signal-transduction proteins such as rhodopsin and β -adrenergic receptors that interact with GTP-binding proteins⁴⁴. So far, however, there is no evidence that reductase forms an ion channel or that it interacts with a G protein (see below).

The function of the conserved and complex membrane-spanning region of reductase was initially obscure as the substrates and products of the enzyme are water-soluble, and only the water-soluble catalytic domain is needed to restore normal growth to an HMG-CoA reductase-deficient line of CHO cells⁴⁵. But unlike the complete enzyme, the degradation of this truncated enzyme is not accelerated by the presence of sterols (Fig. 4). Deletion of two or more membrane-spanning sequences of reductase abolishes sterol-mediated turnover of the native

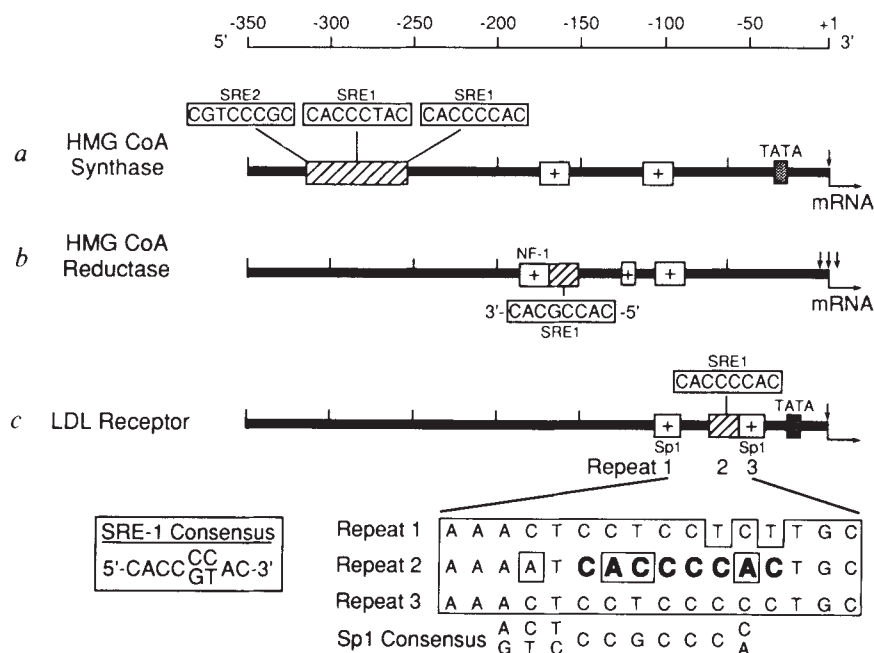


FIG. 3. Sterol regulatory elements (SRE) in the 5' flanking regions of the genes for HMG-CoA synthase (a), HMG-CoA reductase (b) and the LDL receptor (c). +, Sequences that are necessary for transcription in the absence and presence of sterols as identified by linker-scanner mutations. The nucleotide sequence of repeats 1, 2 and 3 in the LDL receptor gene promoter is shown in c. Identical nucleotides are boxed. The core eight nucleotides comprising the SRE-1 in repeat 2 are denoted by bold type. The consensus sequence for transcription factor Sp1⁸⁵ is shown below the repeat 3 sequence.

enzyme⁴⁶ or of a chimaeric enzyme produced by a fusion gene⁴⁷. The sterols therefore seem to act through the membrane-spanning domain of the enzyme.

Evidence now indicates that accelerated degradation of reductase requires a non-sterol isoprenoid as well as a sterol. The early experiments, such as those represented in Fig. 4, were performed with transfected cells that were selected for high expression of reductase by adaptation to compactin (1–5 μ M). So even in the presence of compactin, these cells produced small amounts of mevalonate. Sterols accelerated reductase degradation in part by diverting this mevalonate into a non-sterol regulatory product. In more recent studies, CHO cells were not adapted chronically to an inhibitor, but were incubated acutely with extremely high concentrations of compactin (0.1 mM) that totally block mevalonate production²⁷. Under these conditions sterols alone did not accelerate degradation of the reductase; such acceleration required the addition of exogenous mevalonate, presumably for incorporation into a non-sterol product. The recent discovery of farnesylated proteins raises new mechanistic possibilities in this regard (see below).

In addition to modulating the number of HMG-CoA reductase molecules by 200-fold, cells can inactivate the enzyme reversibly by phosphorylation⁴⁸. This reaction is catalysed by an unusual protein kinase that is stimulated by ADP⁴⁹ or AMP⁵⁰. The same enzyme inactivates acetyl-CoA carboxylase, a rate-controlling enzyme in fatty acid biosynthesis⁵⁰. It is likely that this kinase has a global role in lipid metabolism by inhibiting lipid synthesis when cellular ATP levels fall and ADP and AMP levels rise. There is no evidence that this kinase participates in end-product feedback regulation of mevalonate synthesis.

Regulatory role of oxysterols

For the regulatory actions of LDL to be elicited in repression of cultured cells, there must first be receptor-mediated uptake of LDL, then hydrolysis of LDL-bound cholesteryl esters in lysosomes and, finally, emergence of the cholesterol from the lysosome⁵¹. Genetic blocks at all three of these steps exist in human diseases: familial hypercholesterolaemia⁵¹, cholesteryl ester storage disease⁵¹, and Niemann-Pick type C disease^{53,54}, respectively. Fibroblasts from patients with all three diseases resist regulation by LDL. The blocks can be overcome by adding sterols dissolved in a solvent such as ethanol that allows them to enter the cytosol, bypassing the requirement for receptors or

lysosomes. In such experiments, oxygenated derivatives of cholesterol, such as 25-hydroxycholesterol and 7-ketocholesterol, are up to 100 times more potent than cholesterol itself^{55,56}. These sterols, which are much more water-soluble than cholesterol, achieve higher concentrations in the cytosol, and this may account for their enhanced potency.

The high physiological potency of LDL-derived cholesterol could be attributable to a carrier protein that binds cholesterol when it emerges from the lysosome and escorts it to the regulatory sites. Alternatively, lysosomally liberated cholesterol could be converted into more active oxysterols⁵⁵. A protein that could mediate the regulatory actions of oxysterols has recently been purified⁵⁷, and its cDNA cloned⁵⁸. Originally identified by Kandutsch and co-workers⁵⁹, this cytosolic protein preferentially binds oxysterols that are the most potent metabolic regulators. There is, however, no direct evidence that this protein participates in sterol-mediated regulation, and its metabolic role remains to be defined.

Genetic evidence indicates that LDL-derived cholesterol and solvent-solubilized oxysterols use at least one common step in achieving their regulatory actions. Several classes of mutant CHO cells have been isolated that resist regulation by both agents^{9,60,61}. The mutant cells show defective transcriptional regulation of the three key sterol-regulated genes, but normal stimulation of acyl-CoA: cholesterol acyltransferase⁶¹, which is known to proceed by nontranscriptional mechanisms⁵¹. Single-step revertants simultaneously regain responsiveness to LDL-cholesterol and oxygenated sterols⁶². The mutant cell lines fall into at least two complementation groups, indicating that more than one gene product is required, but so far the gene products have not been isolated. The oxysterol-binding protein seems to be normal in several of these cell lines (P. A. Dawson *et al.*, unpublished observations).

Recently, it was shown that the promoters of all three genes are fully active in two sterol-resistant cell lines from different complementation groups, but that there is no repression by sterols⁶¹. If the SRE-1 elements in the promoters of the LDL receptor and HMG-CoA synthase genes are mutated and transfected into these mutant cells, transcription declines⁶¹. These data indicate that the sterol-resistant mutant cells retain a protein that binds to the SRE-1 and enhances transcription, but that they have lost the ability to silence this protein in the presence of sterols.

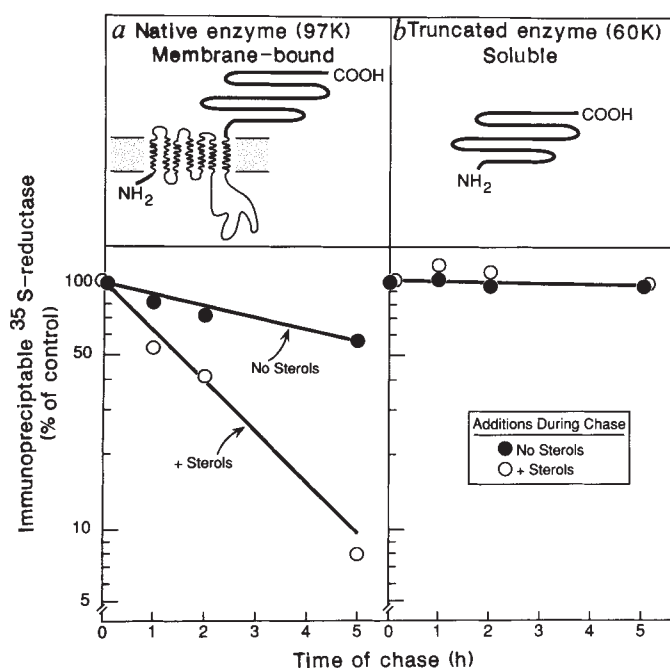


FIG. 4 Degradation of ³⁵S-labelled HMG-CoA reductase in cells transfected with a plasmid encoding the native enzyme (a) or a truncated fragment that contains only the C-terminal soluble (catalytic) domain and lacks amino acids 10 to 341 (b). Reductase-deficient CHO cells⁶³ were transfected with the indicated plasmid under control of the unregulated SV40 promoter⁴⁵. High-expressing cells were selected by growth in medium containing 10% lipoprotein-deficient serum and 1–5 μ M compactin⁴⁵. The cells were pulse-labelled with [³⁵S]methionine in the presence of 40 μ M compactin and absence of lipoproteins. The cells were then chased for the indicated time in the absence of sterols (●) or in the presence of 10 μ g ml⁻¹ cholesterol with 1 μ g ml⁻¹ 25-hydroxycholesterol (○) (see ref. 45 for details). *M*_rs are given in parentheses.

Farnesylated proteins

Linking of mevalonate and cell growth. Cells that are treated with compactin^{7,27,61}, or mutant cells that lack HMG-CoA synthase²⁸ or reductase⁶³, will not grow in the absence of lipoproteins unless large amounts of mevalonate are supplied. The mevalonate requirement is reduced, but not eliminated, by cholesterol supplied in LDL. Remarkably, growth inhibition can be observed in a single cell cycle. When compactin is administered acutely, cells will not enter the DNA synthetic phase (S phase) in response to platelet-derived growth factor without mevalonate⁶⁴⁻⁶⁶. Moreover, cells will not eliminate their reductase activity unless mevalonate is available^{7,26,27}. The non-sterol isoprenoid that allows entry into S phase could be the same as the one that suppresses reductase. But what is it?

Bulk products such as ubiquinone⁶⁷ or dolichol seem unlikely candidates, because cells should have a sufficient storage pool of these metabolites to allow at least one round of cell division. Rather, it seems likely to be a regulatory molecule that must be synthesized at a precise phase of the cell cycle to allow DNA synthesis. A clue emerged in 1984 when Glomset and co-workers discovered that cultured mammalian cells incorporate mevalonate into covalent linkage with proteins of relative molecular mass (M_r) in the ranges of 20,000 to 30,000 (20K-30K) and 50K-70K⁶⁸. A precedent for the covalent isoprenylation of proteins had been established about a decade ago when peptide mating factors secreted by several fungi were shown to contain a farnesyl group attached in thioether linkage to the C-terminal cysteine⁶⁹⁻⁷¹. Subsequent studies with the mating α -factor from *Saccharomyces cerevisiae* and farnesylated proteins from animal cells (see below) have clarified the mechanism of farnesylation. In each of these proteins the farnesylated cysteine is initially the fourth residue from the C terminus (see refs 1, 2 and 72). Immediately after translation, in a sequence of events whose order is not yet totally established, a farnesyl group is attached to this cysteine, the protein is cleaved on the C-terminal side of this residue, and the free COOH group of the cysteine is methylated^{72,73}. All of these reactions are required for the secretion of active α -factor in *Saccharomyces*².

The first mevalonate-labelled animal protein to be identified was lamin B (M_r 67K)^{4-6,74}. Mass spectroscopy analysis showed that this cysteine is covalently attached to a farnesyl group in thioether linkage⁶. The hydrophobic farnesyl moiety could participate in the assembly of the nuclear membrane, and it could also allow lamin B to remain associated with vesicles derived from the nuclear membrane during mitosis. The smaller mevalonate-labelled proteins (M_r 20-30K) are now known to be p21^{ras} proteins, a family of GTP-binding and hydrolysing proteins that regulate cell growth when bound to the inner surface of the plasma membrane^{1,2}. Overproduction of p21^{ras} proteins or mutations that abolish their GTPase activity lead to uncontrolled cell division⁷⁵. All known p21^{ras} proteins contain the cysteine prerequisite, which is processed by farnesylation, proteolysis and COOH-methylation, just as with the yeast mating factor^{1-3,72,73}. The farnesylated p21^{ras} binds loosely to the plasma membrane, from which most of it can be released with salt¹. After binding to the membrane, some p21^{ras} proteins are further modified by the addition of palmitate in thioester linkage to cysteines near the farnesylated C-terminal cysteine¹. Palmitoylation renders the protein even more hydrophobic and anchors it more tightly to the plasma membrane.

An apparently identical pattern of farnesylation and proteolysis occurs in the equivalent yeast proteins, which also contain a cysteine at the fourth position from the C terminus². Elegant genetic studies have demonstrated that the covalently attached farnesyl group is essential to the normal function of yeast Ras proteins in regulating the interplay between nutrient supply and cell division in yeast⁴. The Ras proteins, fungal mating factors, and lamin B all share a similar C-terminal amino-acid sequence^{1,2,73}. This motif consists of an invariant cysteine, followed by two aliphatic amino acids, and a terminal amino acid.

Presumably, this motif functions as the recognition site for a putative farnesyl-protein transferase that is likely to use farnesyl pyrophosphate as the donor.

Regulation of HMG CoA reductase? The studies discussed above establish that farnesylated proteins influence nuclear assembly and cell growth. It is logical that a farnesylated protein would also participate in the feedback control of HMG-CoA reductase, thereby ensuring a steady mevalonate supply. Such a protein would presumably act post-transcriptionally because transcriptional repression is achieved by sterol administration even when farnesylation is prevented²⁷.

When mevalonate is unavailable, proteins that are usually farnesylated accumulate in an unfarnesylated form⁷⁶. Any one of these proteins might be able to alter HMG-CoA reductase through either a positive or negative mechanism operating on translation or degradation. Translational control could be mediated by the interaction of a farnesylated protein with the complex 5' untranslated region of the mRNA or with the membrane-spanning domain of the nascent protein chain. When compactin is added to mammalian cells, reductase first appears on the outer nuclear envelope, indicating that this is the site of translation under conditions of mevalonate deprivation³⁶. The enzyme then moves laterally to the smooth ER, which forms as a projection of the nuclear envelope³⁶. As lamin B is believed to be associated with the inner nuclear membrane, it is not likely to regulate translation of reductase, which could be done, however, by another farnesylated protein on the outer nuclear membrane.

Controlled degradation of reductase is mediated by its membrane-attachment domain⁴⁵⁻⁴⁷, which anchors the enzyme to the nuclear envelope and ER. Farnesylated proteins could influence this degradation by regulating the rate at which reductase is incorporated into vesicles that transport it from the ER to a degradative site. Transport of lipid vesicles from the ER requires a membrane-bound GTP-binding protein⁷⁷, which is likely to be a member of the p21^{ras} family and thus to be farnesylated. Precedent for a role of such proteins in membrane transport comes from the yeast *SEC4*⁷⁸ and *YPT1*⁷⁹ gene products, which are Ras-like proteins that are required for the secretory machinery.

A suggestion of a link between reductase degradation and ER budding comes from studies of UT-1 cells, in which the massively overproduced reductase is housed in an enormously hypertrophied system of smooth ER membranes that form uniform tubules, designated crystalloid ER, which occupy 15% of the cell volume⁸⁰. When UT-1 cells are incubated with sterols, the crystalloid ER disappears along with the reductase within 24 h⁸⁰. As discussed above, sterols may function in part by diverting limited amounts of mevalonate into non-sterol products that include farnesylated GTP-binding proteins of the p21^{ras} family. The seven membrane-spanning regions of reductase are a possible candidate for interaction with such a GTP-binding protein.

Questions

Given the now intense interest in this field and the availability of diverse experimental systems ranging from yeast genetics to mutant human and animal cell lines, there are soon likely to be answers to the following questions:

- Is farnesyl the only mevalonate-derived substance that is covalently attached to proteins? So far, only a small fraction of the [³H]mevalonate-labelled proteins in cells have been analysed. Some of these proteins could contain modified farnesyl residues or other isoprenoid groups.
- Do cell membranes contain receptors for farnesylated proteins? The hydrophobicity of the farnesyl group is not sufficiently specific to explain the selective targeting of lamin B to the nuclear envelope or of Ras protein to the plasma membrane. These membranes presumably contain specific receptors for these proteins. Farnesylation could facilitate this interaction, perhaps by orienting the protein with respect to the membrane

surface. One candidate receptor is the *STE6* gene product, which is required for the unusual post-translational secretion of farnesylated **a**-factor in *Saccharomyces*⁸¹. This protein contains several membrane-spanning regions that could allow it to transport the farnesylated **a**-factor across the plasma membrane.

■ What is the nature of the enzyme that attaches farnesyl groups to proteins? Unusually, it presumably uses a pyrophosphate donor (farnesyl pyrophosphate) to create a thioether bond. Perhaps the closest parallel in animal cells is *S*-adenosyl-L-methionine synthase, which forms a C-S bond using ATP as a donor and splitting off pyrophosphate.

■ Is there only one farnesyl-protein transferase and how is it regulated? And if inhibitors can be found, how will they affect the growth of normal and malignant cells?

Therapeutic implications

The mevalonate pathway has already been exploited in the design of cholesterol-lowering drugs. Inhibition of HMG-CoA reductase by lovastatin and related compounds blocks

cholesterol synthesis and increases transcription of the LDL receptor gene^{82,83}. A variation on this approach has been suggested for cancer chemotherapy². Extremely high levels of reductase inhibitors, given for a short period could prevent farnesylation of Ras proteins, inhibiting the growth of Ras-dependent tumour cells. Inhibitors of the putative Ras farnesyl-protein transferase would be much more specific than HMG-CoA reductase inhibitors in this regard and could be useful on a chronic basis to suppress the growth of certain tumours. It is impossible to predict whether toxicity to normal cells would preclude the use of such an inhibitor, but such toxicity could be overcome by selective targeting of the drug to tumour cells. The recognition of the central role of the mevalonate pathway in cellular metabolism should allow these and other possibilities to be tested in the near future. □

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- Hancock, J. F., Magee, A. I., Childs, J. E. & Marshall, C. J. *Cell* **57**, 1167-1177 (1989).
- Schafer, W. R. *et al. Science* **245**, 379-385 (1989).
- Casey, P. J., Solis, P. A., Der, C. J. & Buss, J. E. *Proc. natn. Acad. Sci. U.S.A.* **86**, 8323-8327 (1989).
- Wolda, S. L. & Glomset, J. A. *J. Biol. Chem.* **263**, 5997-6000 (1988).
- Beck, L. A., Hosick, T. J. & Sinensky, M. J. *Cell Biol.* **107**, 1307-1316 (1988).
- Farnsworth, C. C., Wolda, S. L., Gelb, M. H. & Glomset, J. A. *J. Biol. Chem.* **264**, 20422-20429 (1989).
- Brown, M. S. & Goldstein, J. L. *J. Lipid Res.* **21**, 505-517 (1980).
- Goldstein, J. L. & Brown, M. S. *J. Lipid Res.* **25**, 1450-1461 (1984).
- Chang, T.-Y. & Limanek, J. S. *J. Biol. Chem.* **255**, 7787-7795 (1980).
- Clarke, C. F. *et al. Molec. cell. Biol.* **7**, 3138-3146 (1987).
- Südhof, T. C., Goldstein, J. L., Brown, M. S. & Russell, D. W. *Science* **228**, 815-822 (1985).
- Gil, G., Brown, M. S. & Goldstein, J. L. *J. Biol. Chem.* **261**, 3717-3725 (1986).
- Reynolds, G. A. *et al. Cell* **38**, 275-286 (1984).
- Osborne, T. F., Goldstein, J. L. & Brown, M. S. *Cell* **42**, 203-212 (1985).
- Südhof, T. C., Russell, D. W., Brown, M. S. & Goldstein, J. L. *Cell* **48**, 1061-1069 (1987).
- Südhof, T. C., van der Westhuyzen, D. R., Goldstein, J. L., Brown, M. S. & Russell, D. W. *J. Biol. Chem.* **262**, 10773-10779 (1987).
- Dawson, P. A. *et al. J. Biol. Chem.* **263**, 3372-3379 (1988).
- Osborne, T. F., Gil, G., Goldstein, J. L. & Brown, M. S. *J. Biol. Chem.* **263**, 3380-3387 (1988).
- Smith, J. R., Osborne, T. F., Brown, M. S., Goldstein, J. L. & Gil, G. *J. Biol. Chem.* **263**, 18480-18487 (1988).
- Smith, J. R., Osborne, T. F., Goldstein, J. L. & Brown, M. S. *J. Biol. Chem.* (in the press).
- Gil, G., Osborne, T. F., Goldstein, J. L. & Brown, M. S. *J. Biol. Chem.* **263**, 19009-19019 (1988).
- Gil, G. *et al. Proc. natn. Acad. Sci. U.S.A.* **85**, 8963-8967 (1988).
- Santoro, C., Mermoud, N., Andrews, P. C. & Tjian, R. *Nature* **334**, 218-224 (1988).
- Rajavashisth, T. B., Taylor, A. K., Andalibi, A., Svenson, K. L. & Lusis, A. J. *Science* **245**, 640-643 (1989).
- Endo, A. *Klin. Wschr.* **66**, 421-427 (1988).
- Brown, M. S., Faust, J. R., Goldstein, J. L., Kaneko, I. & Endo, A. *J. Biol. Chem.* **253**, 1121-1128 (1978).
- Nakanishi, M., Goldstein, J. L. & Brown, M. S. *J. Biol. Chem.* **263**, 8929-8937 (1988).
- Panini, S. R., Schnitzer-Polokoff, R., Spencer, T. A. & Sinensky, M. J. *J. Biol. Chem.* **264**, 11044-11052 (1989).
- Chin, D. J. *et al. Proc. natn. Acad. Sci. U.S.A.* **79**, 1185-1189 (1982).
- Luskey, K. L., Faust, J. R., Chin, D. J., Brown, M. S. & Goldstein, J. L. *J. Biol. Chem.* **258**, 8462-8469 (1983).
- Chin, D. J. *et al. Nature* **308**, 613-617 (1984).
- Dynan, W. S. *Trends Genet.* **2**, 196-197 (1986).
- Reynolds, G. A., Goldstein, J. L. & Brown, M. S. *J. Biol. Chem.* **260**, 10369-10377 (1985).
- Hall, R. H. *Prog. Nucleic Acid Res. Molec. Biol.* **10**, 57-86 (1970).
- Liscum, L. *et al. J. Biol. Chem.* **260**, 522-530 (1985).
- Pathak, R. K., Luskey, K. L. & Anderson, R. G. W. *J. Cell Biol.* **102**, 2158-2168 (1986).
- Skalnik, D. G. & Simoni, R. D. *DNA* **4**, 439-444 (1985).
- Luskey, K. L. & Stevens, B. J. *J. Biol. Chem.* **260**, 10271-10277 (1985).
- Basson, M. E., Thorsness, M., Finer-Moore, J., Stroud, R. M. & Rine, J. *Molec. cell. Biol.* **8**, 3797-3808 (1988).
- Gertler, F. B., Chiu, C. Y., Richter-Mann, L. & Chin, D. J. *Molec. cell. Biol.* **8**, 2713-2721 (1988).
- Woodward, H. D., Allen, J. M. C. & Lennarz, W. J. *J. Biol. Chem.* **263**, 18411-18418 (1988).
- Rajkovic, A., Simonsen, J. N., Davis, R. E. & Rothman, F. M. *Proc. natn. Acad. Sci. U.S.A.* **86**, 8217-8221 (1989).
- Learned, R. M. & Fink, G. R. *Proc. natn. Acad. Sci. U.S.A.* **86**, 2779-2783 (1989).
- Dohman, H. G., Caron, M. G. & Leikowitz, R. J. *Biochemistry* **26**, 2657-2664 (1987).
- Gil, G., Faust, J. R., Chin, D. J., Goldstein, J. L. & Brown, M. S. *Cell* **41**, 249-258 (1985).
- Jingami, H., Brown, M. S., Goldstein, J. L., Anderson, R. G. W. & Luskey, K. L. *J. Cell Biol.* **104**, 1693-1704 (1987).
- Skalnik, D. G., Narita, H., Kent, C. & Simoni, R. D. *J. Biol. Chem.* **263**, 6836-6841 (1988).
- Gibson, D. M. in *Regulation of HMG CoA Reductase*. (ed. Priess, B.) 80-132 (Academic, New York, 1985).
- Brown, M. S., Bruntsch, G. Y. & Goldstein, J. L. *J. Biol. Chem.* **250**, 2502-2509 (1975).
- Hardie, D. G., Carling, D. & Sim, A. T. R. *Trends biochem. Sci.* **14**, 20-23 (1989).
- Goldstein, J. L. & Brown, M. S. A. *Rev. Biochem.* **46**, 897-930 (1977).
- Goldstein, J. L., Dana, S. E., Faust, J. R., Beaudet, A. L. & Brown, M. S. *J. Biol. Chem.* **250**, 8487-8495 (1975).
- Pentchev, P. G. *et al. FASEB J.* **1**, 40-45 (1987).
- Liscum, L., Ruggiero, R. M. & Faust, J. R. *J. Cell Biol.* **108**, 1625-1636 (1989).
- Kandutsch, A. A., Chen, H. W. & Heiniger, H.-J. *Science* **201**, 498-501 (1978).
- Krieger, M., Goldstein, J. L. & Brown, M. S. *Proc. natn. Acad. Sci. U.S.A.* **75**, 5052-5056 (1978).
- Dawson, P. A., van der Westhuyzen, D. R., Goldstein, J. L. & Brown, M. S. *J. Biol. Chem.* **264**, 9046-9052 (1989).
- Dawson, P. A., Ridgway, N. D., Slaughter, C. A., Brown, M. S. & Goldstein, J. L. *J. Biol. Chem.* **264**, 16798-16803 (1989).
- Taylor, F. R., Saucier, S. E., Shown, E. P., Parish, E. J. & Kandutsch, A. A. *J. Biol. Chem.* **259**, 12382-12387 (1984).
- Leonard, S. & Sinensky, M. *Biochim. biophys. Acta* **947**, 101-112 (1988).
- Metherall, J. E., Goldstein, J. L., Luskey, K. L. & Brown, M. S. *J. Biol. Chem.* **264**, 15634-15641 (1989).
- Chang, T.-Y. & Chang, C. C. Y. *Biochemistry* **21**, 5316-5323 (1982).
- Mosley, S. T., Brown, M. S., Anderson, R. G. W. & Goldstein, J. L. *J. Biol. Chem.* **258**, 13875-13881 (1983).
- Quesney-Huneuse, V., Wiley, M. H. & Siperstein, M. D. *Proc. natn. Acad. Sci. U.S.A.* **76**, 5056-5060 (1979).
- Habenicht, A. J. R., Glomset, J. A. & Ross, R. J. *J. Biol. Chem.* **255**, 5134-5140 (1980).
- Fairbanks, K. P., Witte, L. D. & Goodman, D. S. *J. Biol. Chem.* **259**, 1546-1551 (1984).
- Maltese, W. A., Aprile, J. R. & Green, R. A. *Biochem. J.* **246**, 441-447 (1987).
- Schmidt, R. A., Schneider, C. J. & Glomset, J. A. *J. Biol. Chem.* **259**, 10175-10180 (1984).
- Kamiya, Y., Sakurai, A., Tamura, S. & Takahashi, N. *Biochem. biophys. Res. Commun.* **83**, 1077-1083 (1978).
- Kamiya, Y., Sakurai, A., Tamura, S. & Takahashi, N. *Agric. Biol. Chem.* **43**, 1049-1053 (1979).
- Sakagami, Y., Yoshida, M., Isogai, A. & Suzuki, A. *Science* **212**, 1525-1527 (1981).
- Gutierrez, L., Magee, A. I., Marshall, C. J. & Hancock, J. F. *EMBO J.* **8**, 1093-1098 (1989).
- Clarke, S., Vogel, J. P., Deschenes, R. J. & Stock, J. *Proc. natn. Acad. Sci. U.S.A.* **85**, 4643-4347 (1988).
- Gruenbaum, Y. *et al. J. Cell Biol.* **106**, 585-596 (1988).
- Gibbs, J. B. & Marshall, M. S. *Micro. Rev.* **53**, 171-185 (1989).
- Repko, E. M. & Maltese, W. A. *J. Biol. Chem.* **264**, 9945-9952 (1989).
- Melancon, P. *et al. Cell* **51**, 1053-1062 (1987).
- Goud, B., Salminen, A., Walworth, N. C. & Novick, P. *J. Cell* **53**, 753-768 (1988).
- Segev, N., Mulholland, J. & Botstein, D. *Cell* **52**, 915-924 (1988).
- Anderson, R. G. W., Orci, L., Brown, M. S., Garcia-Segura, L. M. & Goldstein, J. L. *J. Cell Sci.* **63**, 1-20 (1983).
- McGrath, J. P. & Varshavsky, A. *Nature* **340**, 400-404 (1989).
- Brown, M. S. & Goldstein, J. L. *Science* **232**, 34-47 (1986).
- Ma, P. T. S. *et al. Proc. natn. Acad. Sci. U.S.A.* **83**, 8370-8374 (1986).
- Gil, G., Smith, J. R., Goldstein, J. L. & Brown, M. S. *Proc. natn. Acad. Sci. U.S.A.* **84**, 1863-1866 (1987).
- Kadonaga, J. T., Jones, K. A. & Tjian, R. *Trends biochem. Sci.* **11**, 20-23 (1986).

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The Ailao Shan/Red River metamorphic belt: Tertiary left-lateral shear between Indochina and South China

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The relative importance of thickening and strike-slip extrusion in continental collision is debated. Ductile shear in the Ailao Shan/Diancang Shan metamorphic belt, along the Red River in Yunnan, China, yields outstanding evidence of the latter process. For >500 km, mylonites in this narrow northwest-southeast belt show horizontal lineations on steep, northwest-striking foliation planes, and left-lateral kinematic indicators. U-Pb radiometric ages of ~23 Myr imply that strike-slip movement along this shear zone occurred in the Oligo-Miocene. The strain observed suggests that collision of India with Asia displaced Indochina at least 500 km southeastwards relative to South China.

MUCH insight has been gained into the process of continental collision by studying not only the Himalayan belt but also regions surrounding it to the west and north, such as Afghanistan¹, Kohistan^{2,3} or Tibet⁴⁻⁸ (Fig. 1). By contrast, the tectonics of regions located east of the Himalayas are poorly understood. Although first-order interpretations of the geology of Burma⁹ or southeast Asia^{10,11} have been proposed, the nature, magnitude and age of strains in rocks are generally unknown. Here we report results of fieldwork in Yunnan province, China. Our goal was to characterize and date crustal deformation in and around the Ailao Shan and Diancang Shan mountain ranges, which parallel the Red River fault zone (Fig. 2a), to answer the following questions: how much deformation occurred in the Tertiary? Was it related to the collision between India and Asia? How was it partitioned between overthrusting and strike-slip faulting?

The Red River fault zone

Yunnan province is cut by the NW-SE trending Red River fault zone, which marks the southwestern boundary of the Yangtze platform or South China block^{12,13} (Figs 1 and 2). The fault zone extends over a length of >1,000 km between the gulf of Tonkin and Tibet, and stands out as the most profound discontinuity in the morphology and geology of Yunnan. This is clear either on satellite images, in which the sharply defined fault trace and the narrow trough that guides the Red River (Hong He) show that it is one of the great active faults of Asia¹⁴, or on the geological map of Yunnan, in which it separates regions with different rock types and structural trends¹⁵ (Fig. 2).

South of Midu, the active fault trace hugs the northeastern

flank of the Ailao Shan, a narrow, elongated mountain range with elevations between 2,000 and 3,000 m. Quaternary movement on this segment of the fault zone is now well documented¹⁶. It involves normal and right-lateral slip, often partitioned between two roughly parallel fault strands¹⁶ (Fig. 2b). The range-front fault, or Ailao Shan escarpment is where all of the normal throw occurs. The mid-valley fault, which cuts Neogene sediments in the Red River valley a few kilometres northeast of this escarpment, is purely strike-slip¹⁶. North of Midu, where the Red River fault trace becomes less clear, several more northerly striking active faults have dominant normal throw^{14,16}. The largest fault dips east under the Quaternary basin of Lake Erhai, bounding the eastern flank of the Diancang Shan, a mountain range that towers as much as 4,122 m above Dali (2,019 m, Fig. 2). This relief and the subsidence of the ~1,800-m-deep basin¹⁵ seem to result from continuing normal movement on this fault^{14,16}. Northwest of Diancang Shan, a sharp, linear fault trace similar to that south of Midu continues far towards the northwest (Qiaohou fault, ref. 16) (Fig. 2).

High-grade metamorphic and igneous rocks crop out along the Ailao Shan and Diancang Shan^{12,13,15}, making a narrow structural high (Fig. 2) that continues south into Vietnam¹⁷. These rocks, considered to be mostly Proterozoic in age, have been interpreted as an upthrust slice of the Precambrian basement of the Yangtze platform (ref. 13). To the southwest, the steeply dipping Ailao Shan fault zone separates the high-grade metamorphic core of the Ailao Shan from a belt of lower-grade Palaeozoic schists¹⁵ (Fig. 2). Dismembered outcrops of mafic

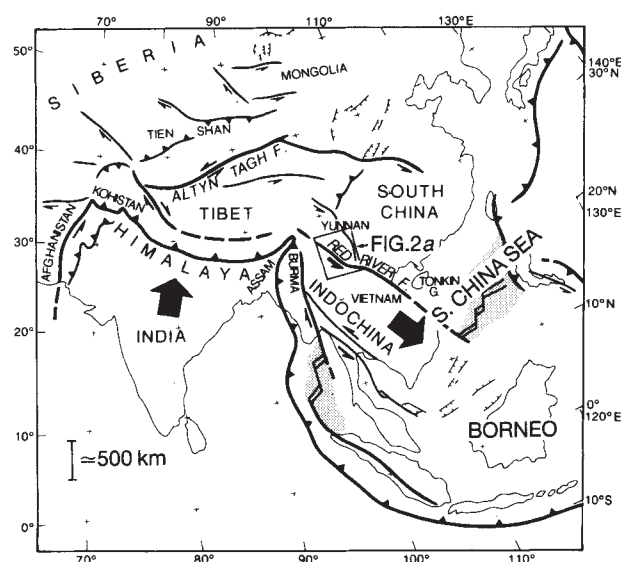


FIG. 1 Schematic map of major Cenozoic fault zones in Asia. Tertiary sea floor is shaded. Redrawn from Fig. 1 in ref. 30.

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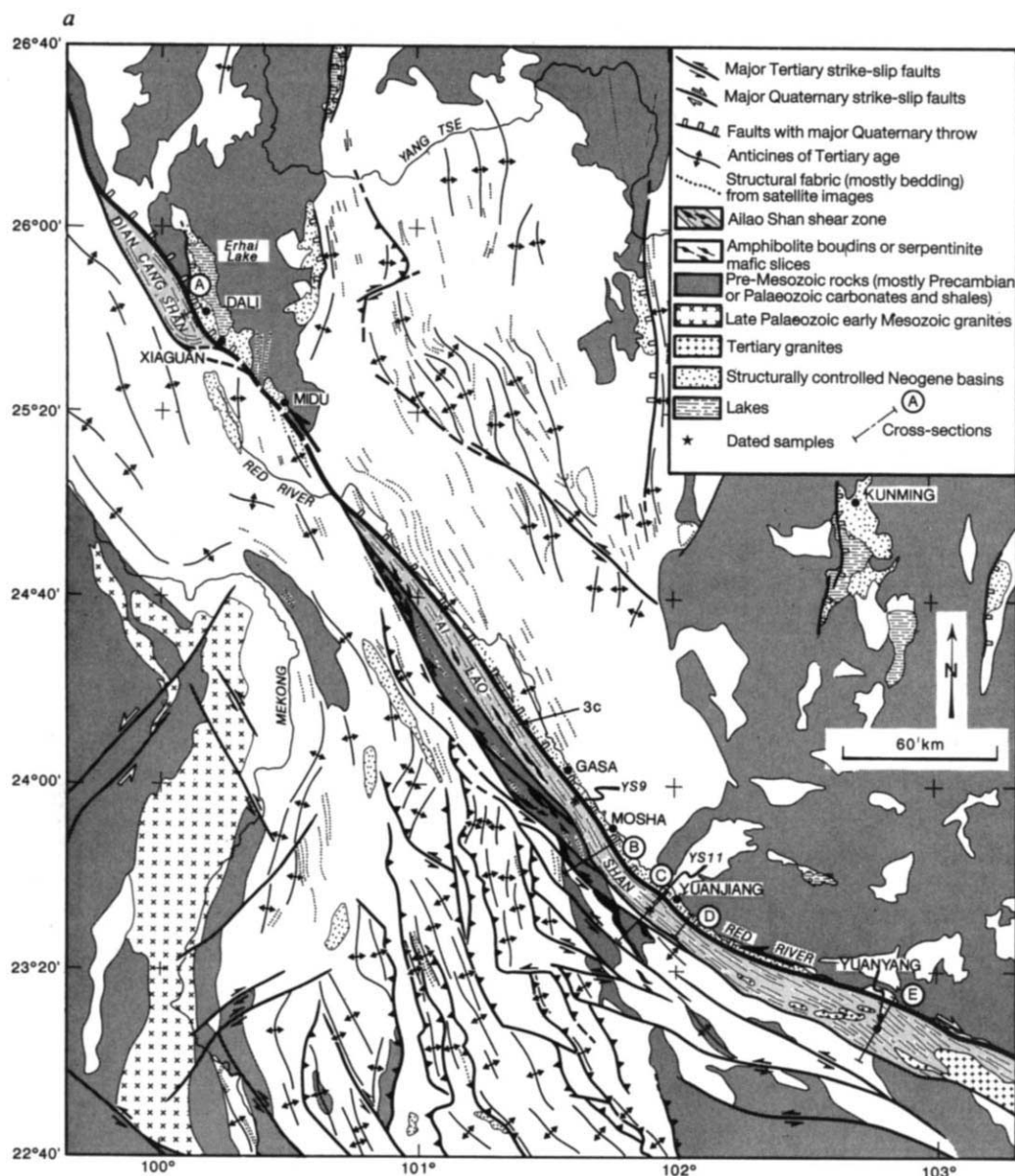


FIG. 2 (Left and opposite) *a*, Map of post Cretaceous tectonics of central Yunnan province. Simplified from ref. 15. *b*, Sections across Ailao Shan and Diancang Shan metamorphic belts. Diagrams on right indicate attitudes of foliation planes and lineation (arrows) in each section. Arrows with numbers locate examples and samples of Figs 3–5.

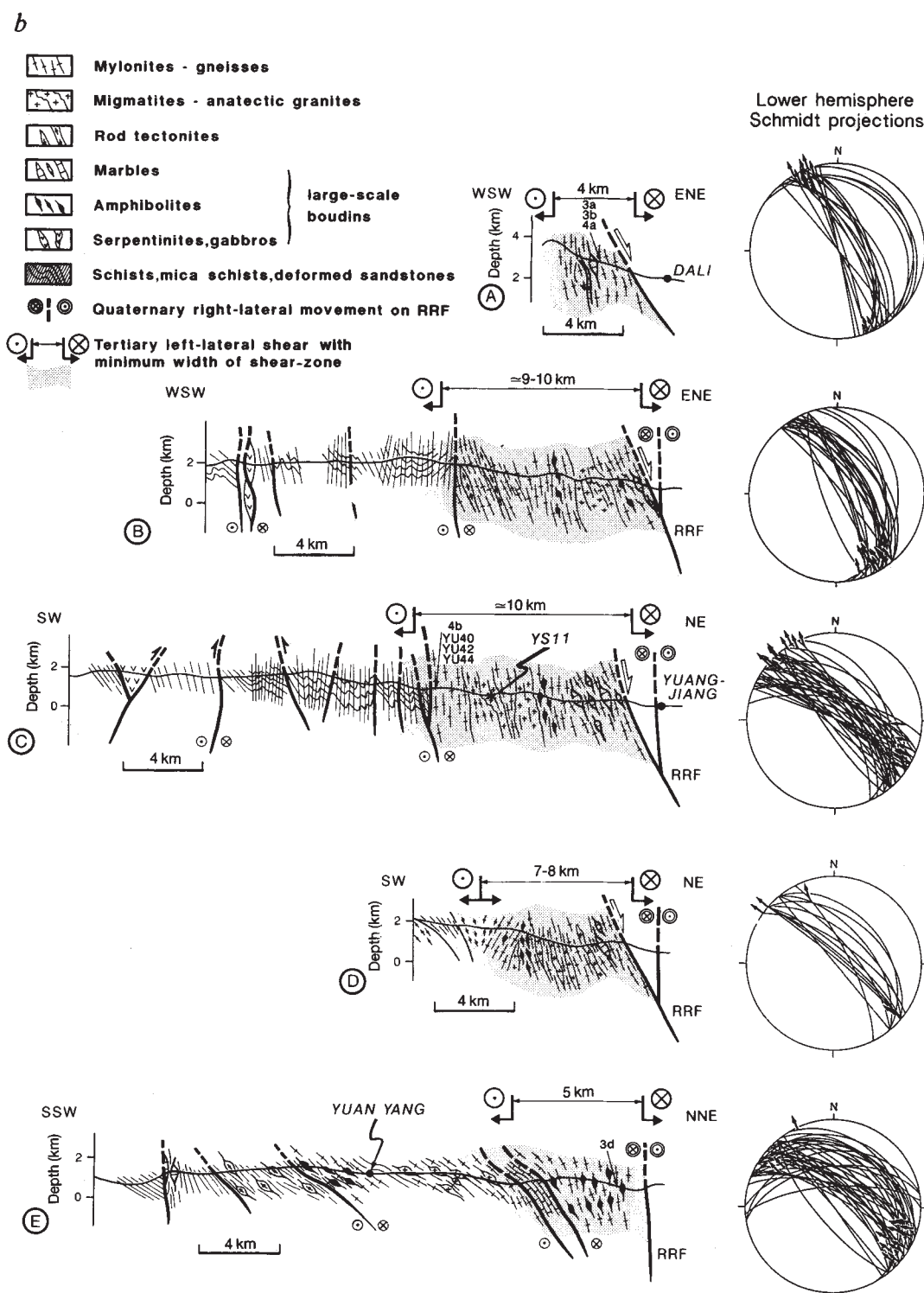
and ultramafic rocks along the southwestern boundary of this latter belt have led various authors to interpret the Ailao Shan as a suture zone, with ages ranging from the early Palaeozoic to the middle Triassic^{10,18–20}.

North of the Ailao Shan/Diancang Shan high, Proterozoic shales and volcanics, capped by Upper Palaeozoic platform carbonates with interbedded basalt flows indicative of an extensional environment in the Permian¹⁵, are covered by an onlapping sequence of mostly continental red sandstones which thickens towards the west into a basin several thousand metres deep. We saw no major unconformity in this sequence, the age of which ranges from the upper Triassic to the Palaeogene¹⁵. The red beds are folded (Fig. 2*a*), often with steep NNW-striking axial cleavage. South of the Ailao Shan, similar red beds have been more strongly shortened, with fold axes and thrust faults striking NW to NNW (Fig. 2*a*). Most of the red beds exposed in this southern basin are of Cretaceous age¹⁵. Farther southwest, along the Mekong (Lancang Jiang) valley, micaschists, mafic volcanics (Palaeozoic?) and a large granite batholith¹⁵ bound the southern Mesozoic basin. This boundary is disrupted by prominent strike-slip faults (Fig. 2).

Over much of Yunnan, Neogene deposits lie in physiographic lows. North of the Red River fault zone, such deposits tend to fill north-south half-graben limited by active normal faults^{14–16}. A narrow trough of Neogene conglomerates follows the Red River valley for >200 km (ref. 15). Southwest of the Ailao Shan, Neogene sandstones are mostly found in the red-bed synclines¹⁵ (Fig. 2*a*). The base of these poorly dated Neogene sediments seems to mark the most important regional unconformity since the Mesozoic.

Ductile, left-lateral shear in metamorphic rocks

The high-grade metamorphic cores of the Ailao Shan and Diancang Shan are made chiefly of steeply dipping, strongly foliated and thinly banded paragneisses (Fig. 3*a, c*). The gneisses are fresh and well exposed (Fig. 3). They include boudins of amphibolite and marbles (Dali marbles), sometimes hundreds of metres wide in sections transverse to the ranges (Fig. 2*a, b*). In the Ailao Shan, such boudins are mostly found near the northeastern boundary of the range. In the Mosha and Yuanjiang sections, which provide the most continuous outcrops across the middle segment of the Ailao Shan, the gneiss core is



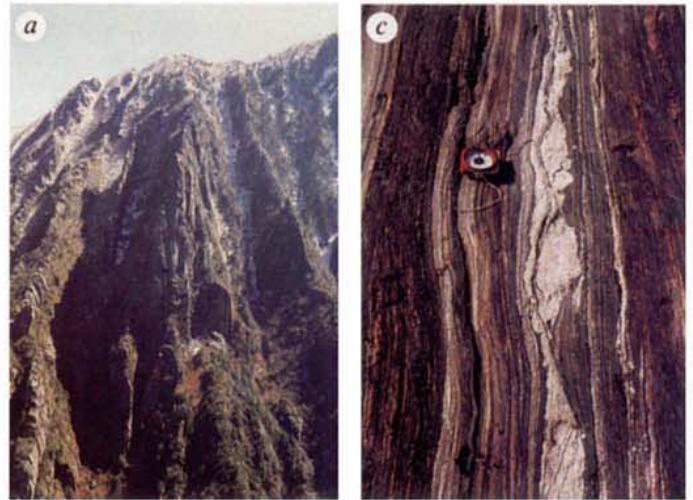
8–10 km wide (B, C, Fig. 2). Its central part is made of biotite augen-gneisses, with migmatites and sheared muscovite-bearing leucogranite bodies elongated parallel to the range (Fig. 2b). The migmatites testify to partial melting, the leucogranites and pervasive pegmatitic dykes that parallel the foliation (Fig. 3c) being a product of crustal anatexis during deformation²¹.

The overall attitude of the gneisses, and the strain in them, are similar in the five sections shown on Fig. 2. The foliation strikes roughly parallel to the local trend of the metamorphic belt, and dips towards the northeast (Fig. 2b). The dips are steep (60–70° NE on the average) in most places (Figs 2 and

3), or even vertical (Fig. 3a). One exception is in the easternmost section of the Ailao Shan (E, Fig. 2), where dips decrease to 30° NE towards the south. Another is in the river gorge west of Xiaguan (Fig. 2a), where the foliation wraps around the southern termination of the Diancang Shan, with dips of 20 to 40° S. Elsewhere, rare gentle dips of the foliation result from local strain heterogeneities, such as boudinage.

The gneisses exhibit a well developed stretching lineation. It is horizontal in the foliation planes in most places (Fig. 3b, d), or locally plunges up to 20° (Fig. 2b). Hence, it strikes nearly parallel to the metamorphic belt. This attitude of the lineation

FIG. 3 *a*, Vertical foliation in gneisses of Diancang Shan, above Dali. View towards southeast. The foliated rock slabs are hundreds of metres high. *b*, Horizontal stretching lineation in paragneisses of Diancang Shan. View towards southwest. *c*, Laminated paragneiss mylonites and boudinage in northern Ailao Shan, north of Gasa. View from above, towards southeast. *d*, Horizontal stretching lineation in gneisses of southeastern Ailao Shan, near Yuanyang. View towards south.

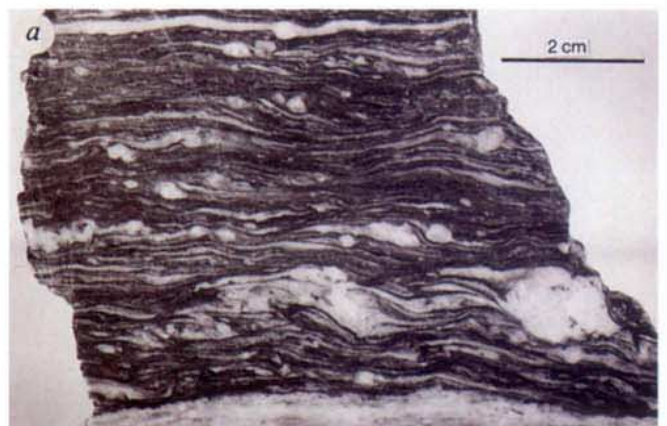


is observed even where the foliation dips less steeply, as in section E (Fig. 2*b*). Some orthogneisses south of the leucogranites in sections B and C display a rod fabric, the lineation being more developed than the foliation. A similar fabric characterizes aplitic orthogneisses along strike in sections D and E, on top of the Ailao Shan.

North of Mosha and Gasa, the Ailao Shan becomes a formidable physiographic barrier, in part because of greater normal throw on the Ailao Shan escarpment¹⁶. In addition, the foliation there is so steep that sharp gneiss crests several kilometres long form the mountain summit, and torrents cascade down gneiss slabs tens of metres high, exposing the horizontal lineation from a distance, as in the Diancang Shan above Dali (Fig. 3*a, b*). Here we found tight sheath folds parallel to the stretching lineation^{22,23}.

In all the sections and sites we visited, the steep foliation and horizontal stretching lineation appear to result from a single phase of non-coaxial, left-lateral strike-slip shear. Abundant kinematic indicators of various types (see, for example, refs 24, 25) are mutually consistent at different scales^{21,22}. On horizontal outcrops polished by streams, asymmetric boudinage of amphibolite and pegmatite sheets parallel to the foliation is widespread, with shear planes between adjacent boudins striking more easterly than the foliation (Fig. 3*c*). North of Mosha and Gasa, boudins only a few tens of centimetres long stand several metres apart, which implies that the late strain recorded by the boudinage is large^{22,24}. Outstanding examples of C-S or C'-S mylonitic fabrics (C and C' being planes of localized shear and S the foliation plane), and of asymmetric tails or rolling structures associated with feldspars are visible in the orthogneisses and migmatitic augen-gneisses (Fig. 4*a, b*), particularly east of Yuanjiang, north of Yuanyang, and above Dali^{21,22}. In thin sections, both microscopic criteria and diagrams of the preferred orientation of quartz *c*-axes (Fig. 4*c*), which display the oblique girdle characteristic of non-coaxial strain (see, for example, refs 24, 25), confirm the left-lateral sense of shear²². The diagram also show a maximum on the *y*-axis of finite strain. This implies that prismatic glide, typical of high temperatures, has occurred²², consistent with the evidence for partial melting in the gneisses²¹.

As in most large shear zones, the finite strain is difficult to assess because only its last increments are measurable in the rocks. Nevertheless, the thinly banded mylonites with marked small-scale composition contrast (Fig. 3*c*), the pervasive asymmetric boudinage of rocks already foliated in earlier phases of the same progressive deformation event, the wide separation of many boudins, and the existence of sheath folds concur to



indicate a large amount of shear²². The shear strain required to form sheath folds and to detach adjacent, foliated boudins is commonly estimated to be of the order of 20 (see, for example, refs 23, 24). We infer this value to be a lower bound for the average strain in the gneiss core of the Diancang Shan/Ailao Shan shear zone²².

Age of the crustal deformation

Rb–Sr or K–Ar ages (for the most part unpublished) obtained so far for metamorphic rocks of the Ailao Shan/Diancang Shan belt range from the late Proterozoic (~840 Myr) to the Tertiary (15–20 Myr). This includes ages of ~230 Myr (late Palaeozoic or early Mesozoic) obtained on muscovites in a gneiss west of Xiaguan.

To clarify this picture and determine the age of the main phase of deformation and metamorphism in the gneisses, we dated the foliation-parallel leucogranitic melts, which are rich in uranium-bearing minerals such as monazite, xenotime and zircon, as is often the case with leucogranites⁵. Figure 5 summarizes our first analytical results²⁶. Seventeen U–Pb analyses were performed on small-size fractions (~0.1 mg) of these three minerals, extracted from samples taken at two sites 50 km apart along the central segment of the Ailao Shan core (YS 9, YS 11, Fig. 2). We discuss the dating results and ²⁰⁶Pb disequilibrium problems in ref. 26. The crystallization ages of the monazite and xenotime fractions at either site cluster between 22 and 24 Myr (Fig. 5). The zircon fractions at one of the sites yield older, discordant ages of about 30 and 34 Myr. Taken with the ages of the monazites and xenotimes, these discordant ages indicate²⁶ that the anatectic melts sampled derive from Upper Precambrian source rocks (Fig. 5).

Given that the leucogranites are less deformed than the gneisses, that a large amount of strain occurred in the gneisses before that now visible on the outcrops, and that melting ought to postdate the onset of strain in shear zones by several million years²⁷, the crystallization ages we found imply that large-scale ductile shear in the Ailao Shan/Diancang Shan gneiss belt took place mostly before 21 Myr. In other words, the early Miocene ages above probably correspond to late phases of a long-lived deformation event.

Further information about the duration and extent of this event may be deduced from the regional stratigraphy and from structural relationships between the metamorphic rocks and the adjacent sediments. As noted earlier, the red-bed series of central Yunnan are mostly conformable from the middle Triassic to the lower Tertiary. Most of the folding of these series thus took place after (not as is often inferred, during) the Mesozoic (Yen-shan phase, refs 12 and 19). The NNW–SSE average trend of the folds (Fig. 2) implies roughly ENE–WSW regional shortening, consistent with left-lateral movement along the Ailao Shan/Red River zone¹¹. The existence of gradients of metamorphism and deformation on either side of the Ailao Shan strengthens this inference. To the north near Gasa, the gradient is particularly large: in less than two kilometres, fracture cleavage in the red beds changes to strong vertical flow cleavage, the rocks become epimetamorphic slates, and the cleavage strike

swings from 170° to 150°, parallel to the foliation in the nearby gneisses²². Going south from Yuanjiang, after a sharp contact between the gneisses and the lower-grade micaschists at the Ailao Shan fault (Fig. 2), the gradient across the Palaeozoic and Triassic is more gradual. Nevertheless, in less than 10 km, the metamorphic grade and the amount of flattening decrease markedly, and the cleavage strike swings from parallel to the Ailao Shan (140°) to nearly north–south. Sheared red beds and slates similar to those near Gasa²² are observed in the gap around Midu where the gneiss cores of the Ailao Shan and Diancang Shan pinch out (Fig. 2). Finally, the source of the Dali marbles, which stretch along the northeastern edge of the Ailao Shan/Diancang Shan belt, seems to be the Upper Palaeozoic carbonates of the Yangtze platform.

Regional folding north and south of the Ailao Shan/Diancang Shan belt, and left-lateral shear along it, thus seem to be two intimately linked, in part successive, manifestations of a Tertiary tectonic event that coherently deformed the Upper Palaeozoic to Palaeogene sedimentary cover and shaped much of the crustal structure of Yunnan. The Eocene age¹⁵ of the youngest red beds involved in folds southwest of the Ailao Shan implies that this event started after 50–60 Myr ago.

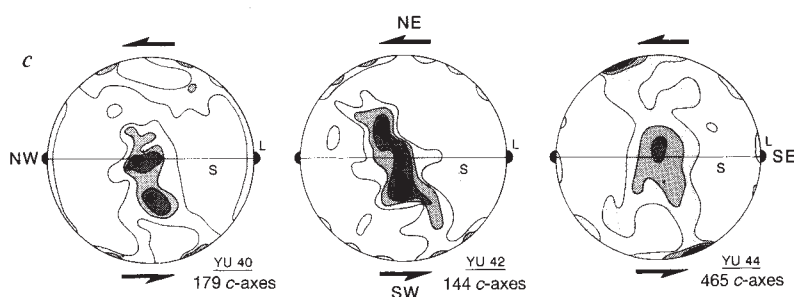
Uplift of the gneisses

Metamorphic mineral assemblages in the paragneisses of the Ailao Shan and Diancang Shan indicate pressures of 400–600 MPa and temperatures of 650–750 °C, in accordance with the occurrence of high-temperature creep in quartz and partial melting^{21,22}. Thus, strike-slip shear in the gneiss cores of these ranges must have taken place at depths of the order of 15–20 km in a rather high geothermal gradient, and the gneisses have been uplifted a minimum of ~15 km since the middle Miocene. Our observations suggest that two different processes have contributed to the uplift.

Along the southwestern edge of the Ailao Shan core, the Ailao Shan fault dips steeply towards the northeast, placing the high-grade gneisses on top of lower-grade schists (Fig. 2). Foliations in the Ailao Shan also dip towards the northeast, the dip becoming shallower near Vietnam. This structure implies that a component of southwest-directed thrust resulting from dominant, although slightly oblique, left-lateral shear could have moved the Ailao Shan core onto the schists, much of this motion being distributed in the schists. Even if the obliquity were slight, a large amount of strike-slip displacement could have produced several kilometres of uplift, the southern limb of the Ailao Shan thus corresponding to a half 'flower structure' of crustal scale.

A second process, compatible with the post-Miocene tectonics of Yunnan, has probably caused a large part of the uplift. In satellite imagery¹⁴ and in the field¹⁶ the Ailao Shan and Diancang Shan escarpments are the most outstanding recent normal faults of central Yunnan, and where they tail out, the gneiss cores of the ranges plunge under the surface sediments (Midu gap, Fig. 2). Normal slip on these faults at rates of ~2 mm yr⁻¹, consistent with field evidence¹⁶, could have produced a structural relief of 3–4 km, comparable to the value obtained by adding the topographic relief of the ranges to the thickness of recent sediments

FIG. 4 (Opposite and right) Left-lateral kinematic indicators: a, Diancang Shan section; b, Yuanjiang section. Views from above. c, Preferred orientation diagrams of quartz c-axes in sections perpendicular to foliation and containing lineation (L), lower hemisphere Schmidt projections. Contours: 1, 2.5, 4, 5.5% per 1% area.



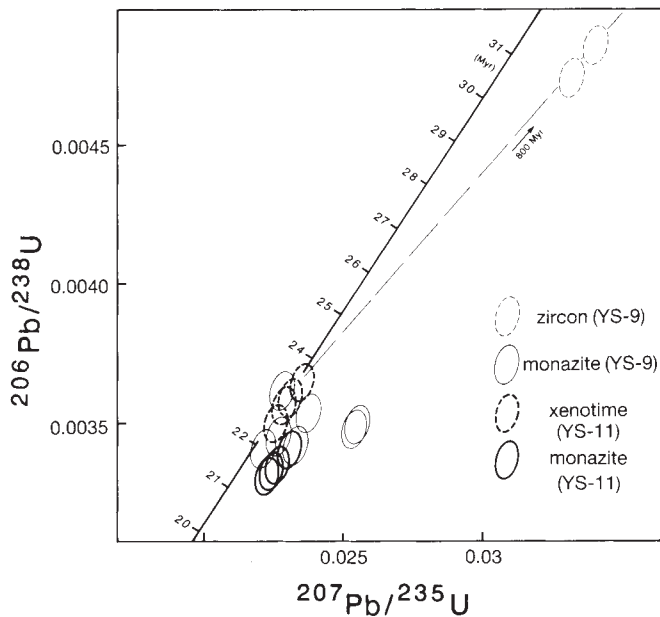


FIG. 5 Concordia diagram for U-Pb analyses of small-size fractions of monazite, xenotime and zircon from two leucogranites (YS 9, YS 11) near Mosha and Yuanjiang. Ellipse size roughly reflects analytical precision on individual isotope ratios. Data from ref. 26.

in the adjacent basins, in less than 2 Myr. Since the upper Miocene, the amount of normal throw on these two faults must have been larger because it is here that the strain and metamorphism gradient are larger and that greenschist, retrograde metamorphism is more developed. Thick chlorite pods mark the traces of the escarpments. Several hundred metres into the gneiss cores, northeast-dipping foliation planes display chlorite-encrusted slickensides indicative of down-to-the-east normal movement. In the river gorge west of Xiaguan (Fig. 2), a component of ductile, down-to-the-east shear on chlorite- and sericite-rich foliation planes dipping an average of 35° towards the east or southeast is observed. The strain has been ductile enough to induce rotation of feldspars and to form quartz ribbons, implying temperatures of ~350 °C (ref. 22). This indicates 7–8 km of uplift by extensional denudation of the Diancang Shan core. Hence, large-scale normal faulting along the north-eastern flanks of the Ailao Shan and Diancang Shan, mostly coeval with recent, roughly east-west, regional extension in Yunnan^{14,16,28}, had an important role in bringing the amphibolite gneiss cores of the two ranges rapidly to the surface. To a degree, both may be viewed as metamorphic-core complexes.

The extrusion of Indochina by continental collision

From our study of the Diancang Shan/Ailao Shan belt and of sections between Kunming and the Mekong (Fig. 2), we conclude that the most prominent phase of crustal deformation in Yunnan occurred during the Tertiary, between the early Eocene (50–60 Myr) and the middle Miocene (15–20 Myr).

To our surprise, we saw no conspicuous unconformity of mildly deformed red beds on strongly folded or foliated Palaeozoic rocks. Where an unconformity is clear north of the Ailao Shan, it seems to be a distal onlap onto a rather stable or block-faulted pre-Upper Triassic platform¹⁵. The early Mesozoic (Indosinian) tectonic phase documented farther south in Indochina¹⁰ has probably affected several areas of Yunnan¹⁹, but it may have been so strongly overprinted by the Tertiary phase that it is hard to recognize. South of the Ailao Shan for instance, the serpentized mafic and ultramafic rocks we saw stand steeply against, and are sheared with, the Triassic red beds in a prominent left-lateral strike-slip fault zone that splays from the Ailao Shan (Fig. 2). Hence, if these serpentized rocks belong to a pre-Upper Triassic suture, that suture was distorted and sheared after the Mesozoic¹¹.

In the mountains along the Red River fault zone ductile strain in gneisses metamorphosed in the amphibolite facies yields evidence for a remarkable Oligo-Miocene episode of left-lateral

shear. Uplifted in part by Plio-Quaternary normal faulting, the gneisses crop out for >500 km across central Yunnan, in the form of elongated range cores only ~10 km wide. On satellite images, the steeply foliated gneisses continue southeast of the Vietnam border to the Red River delta. Thus they form a Tertiary metamorphic belt ≥1,000 km long that reaches the Gulf of Tonkin. Late kinematic crustal anatexis is widespread in the gneisses, pointing to shear heating in the lithospheric mantle as the main heat source and cause of softening and strain localization^{27,29}. Clearly, the belt is a plate-scale, trans-lithospheric zone of localized strike-slip shear (Fig. 1).

The intensity of the strain in the rocks indicates that the left-lateral strike-slip episode of shear we document here dwarfs all other tectonic events along the Ailao Shan/Red River zone, notably the Plio-Quaternary right-lateral motion^{14,16,28}. The active Red River fault trace merely reactivates the crustal discontinuity created by Tertiary movement, the steep foliation in the gneisses guiding the present oblique slip⁶. Outside the Himalayas, the Ailao Shan/Red River shear zone is the only large zone of high-grade Tertiary metamorphism yet discovered in continental eastern Asia. Our study indicates that it represents a strike-slip counterpart of the Himalayan main central thrust. The occurrence of a large southeastward displacement of Indochina relative to South China along the Red River zone during the Tertiary is now beyond doubt (Fig. 1).

The most plausible cause of such plate-scale movement at this time is the India–Asia collision. The penetration of India into the Asian continent must have pushed Indochina southeastwards while rotating it clockwise^{11,30,31} (Fig. 1). The amount of motion thus induced is imprecisely known, but the minimum average shear strain of 20 estimated in the 10-km-wide gneiss core of the Ailao Shan yields a lower bound of 200 km. A displacement of ~500 km is needed to restore, across the Red River zone, the continuity between the Jinsha suture of eastern Tibet³² and the Uttaradit suture of Laos and central Thailand¹⁰, or between the western margins of the Sichuan–Yangtze and Khorat–Kontum platforms¹¹. Note that the amount of Plio-Quaternary right-lateral displacement, which is unknown, must be added to values deduced from matching offset geological features north and south of the Red River to obtain the amount of Miocene left-lateral displacement. Over 500 km of left-lateral movement on the Oligo-Miocene Ailao Shan/Red River shear zone could account for a large part of the opening of the South China Sea^{11,30,31} (Fig. 1), the 700-km-wide sea floor of which seems to have formed mostly between 32 and 17 Myr (ref. 33). The discovery of this zone and of the nature, sense and age of

shear along it verifies predictions made from indentation experiments on plasticine^{30,31}, thereby supporting the idea that strain localization on the scale of the whole lithosphere is one of the

key factors that controls the dynamics of continental tectonics³¹, and that large-scale strike-slip faulting need not be restricted to the late stages of continental collision³⁴. □

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1. Tapponnier, P., Mattauer, M., Proust, F. & Cassaigneau, C. *Earth planet. Sci. Lett.* **52**, 355–371 (1981).
2. Khan Tahirkehl, R. A., Mattauer, M., Proust, F. & Tapponnier, P. *Geodynamics of Pakistan, Spec. Mem. Geol. Surv. Pakistan* 125–130 (1979).
3. Coward, M. P. *et al. Nature* **295**, 22–24 (1982).
4. Allègre, C. J. *et al. Nature* **307**, 17–22 (1984).
5. Schärer, U., Xu Ronghua & Allègre, C. J. *Earth planet. Sci. Lett.* **77**, 35–48 (1986).
6. Armijo, R., Tapponnier, P., Mercier, J. L. & Han Tonglin *J. geophys. Res.* **91**, 13803–13872 (1986).
7. Chang, C.-F. *et al. Nature* **323**, 501–507 (1986).
8. Molnar, P. *et al. Science* **235**, 299–305 (1987).
9. Mitchell, A. H. G. *J. geol. Soc. Lond.* **138**, 109–122 (1981).
10. Sengor, A. M. C. *Spec. Pap. geol. Soc. Am.* No. 195, 1–88 (1984).
11. Tapponnier, P., Peltzer, G. & Armijo, R. *Spec. Publ. J. geol. Soc. Lond.* No. 19, 115–157 (1986).
12. Zhang, W.-Y. *et al. The Marine and Continental Tectonic Map of China and its Environs* Scale 1:5,000,000 (Science, Beijing, 1983).
13. Cheng, Y.-K. *Yunnan Geology* **6**, 291–297 (1987).
14. Tapponnier, P. & Molnar, P. *J. geophys. Res.* **82**, 2905–2930 (1977).
15. Academia Sinica *Geologic Map of Yunnan* Scale 1:1,000,000 (Science Press, Beijing, 1981).
16. Allen, C. R. *et al. Bull. geol. Soc. Am.* **95**, 686–700 (1984).
17. *Geologic Map of North Vietnam* Scale 1:1,000,000 (Res. Inst. Geol. Min. Resources of Vietnam, Hanoi, 1979).
18. Duan, X.-H. & Zhao, H. *Acta geol. Sinica* **55**, 4 (1981).
19. Li, C.-Y., Wang, Q.-A., Lin, X.-Y. & Tang, Y.-Q., *Tectonic Map of Asia, and Explanatory Notes to the*

- Tectonic Map of Asia* Scale 1:8,000,000 (Chin. Acad. of geol. Sci., Cartographic Publishing House, Beijing, 1982).
20. Bally, A. W. *et al. U.S. geol. Surv. Open File Rep.* **80-501** (1980).
21. Ji, S.-C. *Sci. geol. Sinica* **4**, 347–356 (1988).
22. Leloup, P. H. *et al.* (in preparation).
23. Lacassin, R. & Mattauer, M. *Nature* **316**, 739–742 (1985).
24. Gaudemer, Y. & Tapponnier, P. *J. struct. Geol.* **9**, 159–180 (1986).
25. Lacassin, R. *Tectonics* **6**, 69–88 (1987).
26. Schärer, U. *et al. Earth planet. Sci. Lett.* (in the press).
27. Fleitout, L. & Froidevaux, C. *J. struct. Geol.* **2**, 159–164 (1980).
28. Le Dain, A. Y., Tapponnier, P. & Molnar, P. *J. geophys. Res.* **89**, 453–472 (1984).
29. Poirier, J. P. *J. struct. Geol.* **2**, 135–142 (1980).
30. Tapponnier, P., Peltzer, G., Le Dain, A. Y., Armijo, R. & Cobbold, P. *Geology* **10**, 611–616 (1982).
31. Peltzer, G. & Tapponnier, P. *J. geophys. Res.* **93**, 15085–15117 (1988).
32. Wang, E. & Chu, J. J. *Tectonophysics* **147**, 71–84 (1988).
33. Taylor, B. & Hayes, D. E. *Am. geophys. U. Monogr.* Vol. 27, Pt 2, 23–56 (1983).
34. England, P. C. & Houseman, G. A. *J. geophys. Res.* **91**, 3664–3674 (1986).

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Factor required for mammalian spliceosome assembly is localized to discrete regions in the nucleus

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A monoclonal antibody raised against mammalian spliceosomes specifically recognizes a non-snRNP factor required for spliceosome assembly. This splicing factor is highly concentrated in discrete regions within the nucleus, in a pattern that is a distinct subset of that seen with anti-snRNP antibodies. These observations are evidence that spliceosome assembly could be compartmentalized within the nucleus.

NUCLEAR pre-messenger RNA splicing takes place in spliceosomes, which are composed of the small nuclear ribonucleoprotein particles (snRNPs) U1, U2, U4, U5, U6 and other splicing factors^{1,2}. A number of splicing activities have been identified by *in vitro* complementation³ and by biochemical fractionation of nuclear extracts^{3–7}. In addition, several proteins that specifically bind pre-mRNA have been identified^{8–12}. The study of these splicing factors and RNA-binding proteins has until now been impeded by difficulties in obtaining adequate amounts of purified protein.

In this study we use monoclonal antibodies against partially purified spliceosomes to identify mammalian splicing factors. Functional spliceosomes have previously been prepared by fractionating large-scale splicing reactions on a gel filtration column^{13,14}: fractions containing spliceosomes are highly enriched in the five snRNPs required for splicing and in a subset of nuclear proteins¹³. We use these enriched spliceosome fractions to generate monoclonal antibodies against individual components of the spliceosome. A number of these antibodies bind

to factors associated with spliceosomes, and one, α SC-35 (anti-splicing component, relative molecular mass 35,000 (35K)) specifically recognizes a 35K non-snRNP protein required for *in vitro* pre-mRNA splicing and for spliceosome assembly. The α SC-35 therefore provided us with the opportunity to localize an individual non-snRNP splicing component within the nucleus. Previous studies have shown that snRNP antigens are concentrated in discrete regions within the nucleus and are also present in a diffuse pattern in the surrounding nucleoplasm^{15–19}. We show that the SC-35 antigen localizes with snRNP antigens in the concentrated regions, but not in the surrounding nucleoplasm. Thus, the active splicing apparatus may be localized in specific nuclear structures.

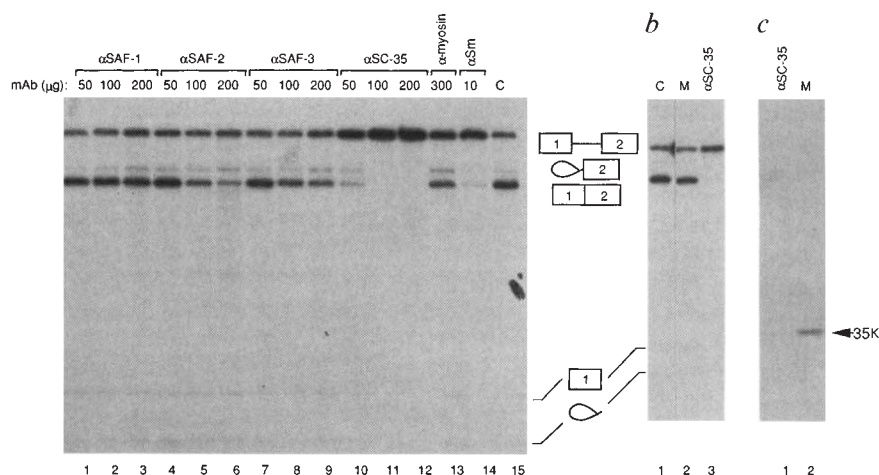
Characterization of monoclonal antibodies

Spliceosomes blocked at a step between spliceosome assembly and the first step of the splicing reaction¹⁴ were fractionated on a Sephacryl S-500 column^{13,14} and used to raise mouse monoclonal antibodies. The resulting hybridomas were screened by enzyme-linked immunosorbent assay (see the legend to Fig. 1). Antibodies purified from positive clones were then tested for their ability to inhibit splicing *in vitro*: it was found that α SC-35 strongly inhibited splicing (Fig. 1a, lanes 10–12), whereas others such as the antibody α SAF-1 (spliceosome associated factor; lane 1–3) had no effect. There was some inhibition by antibodies α SAF-2 (lanes 4–6) and α SAF-3 (lanes 7–9), but only when large amounts were used. The anti-spliceosome antibodies were also tested for their ability to deplete splicing activity from nuclear extracts, and only α SC-35 tested positive (Fig. 1b). Western blot analysis revealed that α SC-35 binds to a 35K protein in untreated extracts, but that the amount of this protein

FIG. 1 The effect of anti-spliceosome monoclonal antibodies (mAbs) on pre-mRNA splicing *in vitro*. **a**, Inhibition of *in vitro* splicing. T7-H β pre-mRNA was incubated in the standard splicing reaction in the presence of purified mAbs. Lane 1–12, splicing with increasing amounts of four different mAbs, as indicated; lane 13, negative control for splicing inhibition with α -myosin mAb (a gift from D. Kiehart⁴²); lane 14, positive control for splicing inhibition with an α Sm mAb (Y12; gift from J. A. Steitz¹⁵); lane 15, splicing reaction without mAb. Splicing was significantly inhibited with 50 μ g α SC-35 antibody, and completely abolished with 100 μ g. For comparison, we show that there is also significant inhibition with 10 μ g of an α Sm mAb (lane 14). The structures are indicated for precursor RNA, lariat intermediate, spliced product, released exon 1 and lariat. **b**, Immunodepletion of splicing activity with immobilized α SC-35 mAb. An α SC-35 immunoaffinity column was prepared

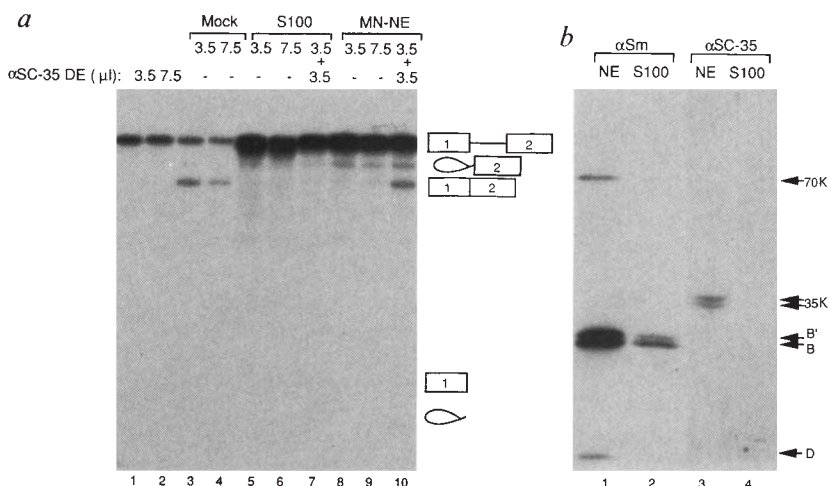
(see methods). Lane 1, splicing reaction with untreated nuclear extracts; lane 2, mock-depletion; lane 3, SC-35-depletion. **c**, A western blot of the SC-35 antigen remaining in mock-depleted and SC-35-depleted nuclear extracts. Aliquots of each extract (~150 μ g total protein of each) were fractionated by 10% SDS-PAGE, transferred to nitrocellulose, and probed with α SC-35 (20 μ g ml⁻¹) followed by ¹²⁵I-labelled anti-mouse immunoglobulin (Amersham). Lane 1, SC-35 depleted extract; lane 2, mock-depleted extract.

METHODS. the 60S spliceosomes were prepared as described¹⁴ and fractionated on a Sephacryl S-500 column^{13,14}. Fractions were pooled and precipitated in 80% cold (-20 °C) acetone. The precipitate was solubilized in 0.1M NaOH, neutralized with 0.1 volumes of 1M HCl and diluted with PBS to a protein concentration of 200 μ g ml⁻¹ (Bradford assay⁴⁰). Female RBF-DNJ mice (Jackson) and FOX-NY myeloma cells⁴¹ were used to make hybridomas⁴² which were screened by enzyme-linked immunosorbent assay (ELISA) using partially purified spliceosome as antigen, and then the antibody subtypes were determined using kits from Bio-Rad. Of 300 hybridomas, 22 tested positive in the ELISA, and 16 of them were successfully cloned. The subtype of α SC-35 was IgG1 class. The supernatants from these clones were tested for their ability to immunoprecipitate spliceosomes. Fifteen supernatants tested positive in this assay. Five of these, including α SC-35, could precipitate spliceosomes as efficiently as an α Sm mAb, but the rest were less efficient (data not shown). Monoclonal antibodies were purified from ascites using anti-mouse IgG affinity columns (Cappel). For *in vitro*



inhibition assays, purified antibodies were concentrated to about 35 mg ml⁻¹ in nuclear extract dilution buffer (20 mM HEPES, pH 7.6, 100 mM KCl). Aliquots from 1.5 to 7.5 μ l were used in standard splicing reactions (25 μ l) containing 10 ng of ³²P-labelled T7-H β pre-mRNA and 7.5 μ l nuclear extract (20 mg ml⁻¹ total protein) prepared as previously described¹⁴. For immunodepletion assays, we used affinity columns with 0.1 ml Affi-gel 10 resin (Bio-Rad) and 3 mg purified antibody, according to the manufacturer's instruction. The coupled resins were transferred to centrifuge filter units (Costar), in which filter membranes are replaced by porous polyethylene bed-support discs. The columns were equilibrated in nuclear extract dilution buffer. Before depletion, the buffer was removed by centrifugation at 2,000 r.p.m. for 2 min in a microcentrifuge. Significant depletion can be achieved by directly passing nuclear extracts through an α SC-35 immunoaffinity column, but is more efficient if nuclear extracts are preincubated with ATP. Incubation of extracts with ATP may lead to the release of antigen from endogenous splicing complexes, or aggregates. In a typical depletion experiment, the splicing reaction mixture (100 μ l) was incubated with 30 μ l nuclear extract at 30 °C for 30 min, applied to the affinity column and incubated at 4 °C for 30 min. The depleted mixture was collected by centrifugation. The splicing reaction was initiated by adding 10 ng ³²P-labelled H β pre-mRNA. After incubation for 90 min, reaction products and intermediates were analysed by electrophoresis on denaturing 6% polyacrylamide gels. The column was regenerated by washing with 0.1 M triethylamine (pH 11.5) to remove bound antigen.

FIG. 2 *In vitro* complementation of SC-35-depleted nuclear extracts. **a**, Splicing reactions were in 3.5 μ l or 7.5 μ l of SC-35-depleted extract (designated α SC-35 DE) (lanes 1 and 2), mock-depleted extract (lanes 3 and 4), S100 extract (lane 5 and 6), or micrococcal nuclease-treated nuclear extract (MN-NE) (lanes 8 and 9). *In vitro* complementation assays: 3.5 μ l α SC-35 DE + 3.5 μ l S100 extract (lane 7), 3.5 μ l α SC-35 DE + 3.5 μ l MN-NE (lane 10). There was no splicing activity with the SC-35-depleted extract alone, and only a low level with the MN-NE alone. When equal volumes of the two extracts were mixed, there was a significant increase in the level of splicing (lane 10). No splicing was observed in the S100 extract alone, or in a mixture of the SC-35-depleted extract and S100 (lane 7). **b**, Detection of snRNP antigens and the SC-35 antigen in nuclear extract (NE) and S100 extract by western blotting analyses. Equivalent amounts (~200 μ g total protein) of nuclear extracts (lanes 1 and 3) and S100 (lanes 2 and 4) were fractionated on a 10% SDS-PAGE, transferred to nitrocellulose and probed with α Sm (lanes 1 and 2) or with α SC-35 (lanes 3 and 4). **METHODS.** Standard splicing reactions were assembled in separate tubes



using equivalent amounts of MN-NE (ref. 3), S100, and SC-35-depleted NE. Aliquots (12.5 μ l) of each reaction mixture were mixed, incubated and electrophoresed on denaturing 6% polyacrylamide gels.

is significantly lower in immunodepleted extracts (Fig. 1c). Thus, the loss of splicing activity is correlated with a reduction in the level of the SC-35 antigen.

We also characterized the SC-35 antigen by *in vitro* complementation. As shown in Fig. 2a, SC-35-depleted extracts could be complemented with a micrococcal nuclease-treated nuclear extract, but not with an S100 extract. All of the main snRNPs, except U5, are selectively inactivated in the nuclease-treated nuclear extract^{3,20}, whereas the S100 extract contains functional snRNPs but lacks a very small subset of splicing factors^{3,8,21}. If the 35K protein corresponds to the splicing factor that is missing from the antibody-depleted extracts, it should be present in the nuclear extracts, but not in the S100. This prediction was confirmed by showing that α SC-35 detects a 35K protein in nuclear extracts, but not in S100 extracts (Fig. 2b, lanes 3 and 4). In parallel experiments, an anti-Sm antibody (α Sm) detects four characteristic proteins in both the nuclear and S100 extracts (Fig. 2b, lanes 1 and 2), although substantially more of these antigens are found in the nuclear extracts.

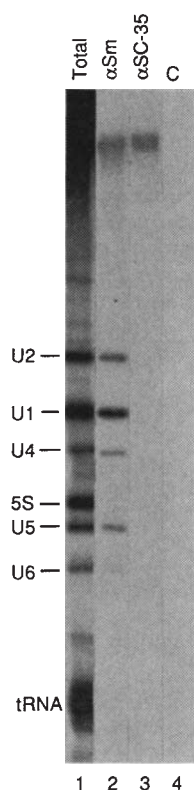


FIG. 3 The SC-35 antigen is not tightly associated with snRNPs. Nuclear extracts were prepared from HeLa cells cultured with 32 P-labelled inorganic phosphate. 32 P-labelled nucleic acids from total extract (lane 1), or from immunoprecipitates with α Sm (lane 2), α SC-35 (lane 3), or control beads containing no antibody (lane 4) were extracted, fractionated on a denaturing 7% polyacrylamide gel, and autoradiographed.

METHODS. HeLa cells (in one 150 mm plate) were washed twice with PBS and starved for 30 min in 15 ml phosphate-free medium (HEPES-KOH, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 5 mM KCl, 2 mM glutamine, 1.8 mM glucose, 2% fetal bovine serum, and 1 $\times$ penicillin-streptomycin). The starved cells were incubated overnight in 15 ml phosphate-free medium and 1 mCi 32 Pi (NEN). The cells were collected in 10 ml NP-40 lysis buffer (10 mM Tris, pH 7.5, 140 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, and 0.5% NP-40) and homogenized, then centrifuged to collect nuclei. The nuclei were extracted in 1 ml buffer (10 mM Tris, pH 7.5, 0.3 M NaCl, 1 mM $MgCl_2$, 0.2 mM EDTA) for 1 h at room temperature and the extract cleared by centrifugation at 4,000 r.p.m. for 10 min. Aliquots of 0.1 ml were immunoprecipitated with 10 μ g mAb coupled to anti-mouse IgG beads (Cappel) in 1 ml IP_{150} (10 mM Tris, pH 7.6, 150 mM NaCl, 1% Triton X-100). Nucleic acids extracted from the immunoprecipitates were analysed on 7% acrylamide-urea denaturing gels and autoradiographed.

The data in Fig. 2b also show that the 35K protein does not correspond to any of the known Sm antigens found in snRNPs (70K, 29K B', 28K B, and 16K D proteins)²². But the SC-35 antigen has a molecular weight similar to other snRNP proteins such as the 33K U1 snRNP-specific A protein, or the 32K U2 snRNP-specific A' protein²². These proteins co-fractionate with snRNPs on columns^{22,23} and on CsCl gradients⁸. To investigate the possible association of the SC-35 antigen with snRNPs, we used α SC-35 for immunoprecipitation. We found that snRNPs labelled with 32 P *in vivo* were efficiently immunoprecipitated with α Sm and α U1 antibodies, but not with α SC-35 (Fig. 5). Consistent with these results was the fact that α SC-35 could not immunoprecipitate any snRNP (as monitored by 32 P-pCp-labelling) either directly from nuclear extracts or purified from a CsCl gradient (data not shown). We conclude that the 35K protein is not tightly associated with snRNPs and therefore does not correspond to any known snRNP structural protein.

The SC-35 antigen has properties similar to a splicing factor designated SF2 (ref. 3): both factors are required for the first step of the splicing reaction, are resistant to micrococcal nuclease, and are present in nuclear, but not S100, extracts³. Pure SF2 migrates as a doublet on an SDS-polyacrylamide gel, with a molecular weight similar to that of SC-35 (A. Krainer, personal communication). Experiments are in progress to determine whether SC-35 and SF-2 are the same factor.

SC-35 antigen and spliceosome assembly

Nuclear extracts from which the SC-35 antigen has been depleted have no splicing activity (Fig. 1b), indicating that this antigen is required for the first step of the splicing reaction. To determine whether the SC-35 antigen is required before or after spliceosome assembly, we analysed the effect of α SC-35 on the formation of splicing complexes by density gradient sedimentation (Fig. 4) and by native gel electrophoresis (Fig. 5).

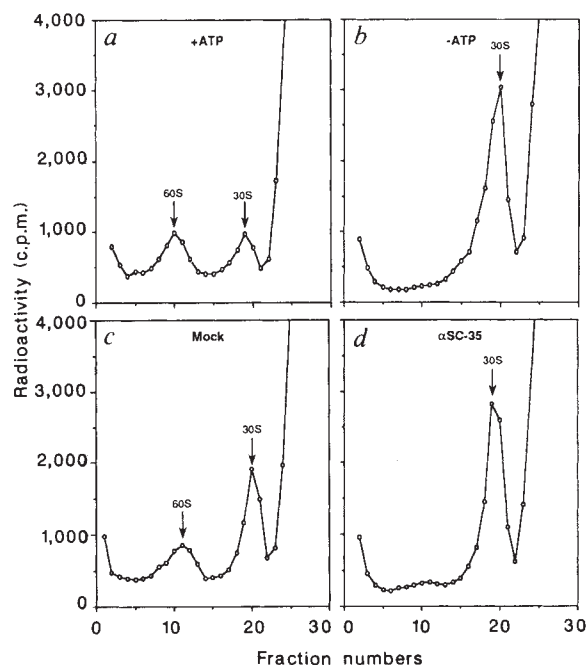


FIG. 4 The SC-35 antigen is required for the formation of 60S splicing complex. 32 P-labelled T7-H β pre-mRNA was incubated with untreated nuclear extracts under splicing conditions with (a) or without (b) ATP at 30 $^{\circ}$ C for 40 min. These reactions served as controls for the formation of 60S spliceosome. The labelled pre-mRNA was also incubated with mock-depleted (c) or SC-35-depleted (d) nuclear extracts under the same conditions. Reaction mixtures were sedimented through 10–30% glycerol gradients, and the fractions collected and counted¹³. The positions of the 30S complex and the 60S spliceosome are indicated.

In agreement with previous studies^{24,25}, both 30S and 60S complexes are observed when labelled pre-mRNA is incubated in untreated nuclear extracts in the presence of ATP (Fig. 4a), whereas only the 30S complex is found in the absence of ATP (Fig. 4b). Both complexes are also seen with ATP present when pre-mRNA is incubated in extracts that have been mock-depleted with a control antibody (Fig. 4c); however, the 30S, but not the 60S, complex is observed in SC-35-depleted extracts when ATP is present. We conclude that the SC-35 antigen is required for assembly of the 60S spliceosome complex, but does not affect the formation of the 30S complex. This conclusion is supported by our finding that α SC-35 can immunoprecipitate 60S spliceosomes blocked both before and after the first step of the splicing reaction^{13,14} (data not shown), indicating that the association of the SC-35 antigen with the spliceosome persists at least through cleavage at the 5' splice site and lariat formation.

Fractionation of ³²P-labelled splicing complexes by electrophoresis on native gels results in discrete complexes designated H, A and B (refs 26, 27). The B complex corresponds to the 60S spliceosome and the 30S complex probably corresponds to a mixture of uncharacterized RNP complexes, including H complexes^{25,27,28}. We investigated whether the SC-35 antigen is necessary for the formation of these complexes by electrophoresis of pre-mRNA incubated in the SC-35-depleted nuclear extracts. As expected, both the A and B complexes were found in untreated extracts with ATP (Fig. 5, lane 2) and only the H complex was detected when pre-mRNA was incubated without ATP (Fig. 5, lane 1). When the pre-mRNA was incubated in a mock-depleted extract, the kinetics were typical of A and B complex formation (Fig. 5, lanes 3–8; see ref. 26 for comparison). By contrast, formation of both the A and B complex was significantly reduced in the SC-35-depleted nuclear extracts (Fig. 5, lanes 9–14). We conclude that the SC-35 antigen is required for assembly of both the A and B complexes.

It has been proposed that the 30S complex and the A complex are spliceosome precursors (so-called pre-spliceosome^{25,26}), but there is no direct evidence as yet that they are functional intermediates. The A complex, which contains U2 snRNP, requires U2AF for its formation: U2AF was originally defined as a

micrococcal nuclease-resistant activity required for U2 snRNP binding to the branchpoint sequence⁸. U2AF activity binds to the 3' splice site and is present in nuclear, but not S100 extracts⁸. The SC-35 antigen has similar properties, but we do not know whether it binds to pre-mRNA. In a separate study, two column fractions, SF*1 and SF*3, were shown to be required for A complex formation²⁹ (the asterisk distinguishes splicing factors identified by Kramer *et al.*^{4,7,29} from those reported by Krainer and Maniatis³). SF*1 is a mixture of protein factors and U2 snRNP, whereas SF*3 is a protein factor(s)²⁹ which behaves chromatographically like U2AF (ref. 8). Thus, we cannot yet distinguish the SC-35 antigen, U2AF, from SF*3, and indeed they may turn out to be one factor.

Nuclear localization of SC-35 antigen

Earlier immunofluorescent staining with anti-snRNP antibodies has shown that snRNPs are concentrated into 20–50 regions within the nucleus, and that they are also present in lesser amounts in the surrounding nucleoplasm^{15–19}. It has been suggested that splicing activity could be restricted to those regions where the snRNPs are concentrated^{18,30–32}, but these could also be sites of snRNP assembly or storage, and the snRNPs active in splicing could be distributed uniformly throughout the nucleoplasm. It is also possible that both the concentrated and diffuse regions of snRNPs are active in splicing.

The SC-35 antigen is a non-snRNP protein which is required for splicing activity, so a comparison of its localization with that of snRNPs in the nucleus may help explain the significance of the speckled and diffuse staining patterns seen with anti-snRNP antibodies. As shown in Fig. 6a, the α SC-35 antibody detects discrete speckles, and the α Sm and α U1 antibodies give a similar speckled pattern, but on a diffuse background (Fig. 6b and d; see also ref. 33). Antibody titration confirms that these different staining patterns do not result from variations in concentrations or affinity of the antibodies used to stain the cells. At low concentrations of polyclonal and monoclonal α Sm or α U1 antibodies (1–5 μ g ml⁻¹), we observe both the speckled and diffuse patterns, but at higher concentrations (10–50 μ g ml⁻¹) we see only the diffuse pattern (data not shown, and ref. 15). However, α SC-35 gave only the speckled pattern at all concentrations between 1 μ g ml⁻¹ and 100 μ g ml⁻¹.

Considered together, these observations indicate that spliceosomes containing SC-35 are concentrated in discrete regions within the nucleus. To establish whether the α SC-35 and anti-snRNP antibodies actually stain the same regions within a single nucleus, we used indirect double immunofluorescence with α SC-35 (mouse origin) and human α Sm and α U1 sera. As shown in Fig. 6b–e, the discrete regions revealed by the three antibodies are identical. Therefore, the SC-35 antigen is localized with snRNPs only in specific compartments within the nucleus. We have also examined the immunofluorescent staining pattern with other antibodies that have no effect on splicing, as assayed by immunodepletion, and none produces the speckled pattern.

This compartmentalization of spliceosomes within the nucleus could be important to the relationship between transcription and pre-mRNA processing. Electron microscopy of nascent RNA transcripts reveals spherical particles at sites corresponding to splice junctions, indicating that spliceosomes might be assembled on pre-mRNA while it is being transcribed^{34,35}. Immunofluorescence microscopy of amphibian oocyte nuclei has shown that snRNPs are present in lampbrush chromosome loops in the form of 20 μ m spheres³⁶; these spheres are attached to specific chromosomal loci and are also free in the nucleoplasm. The snRNPs are also distributed along the RNA fibres extending from the lampbrush chromosomes. These observations have led to the proposal that the spheres are sites of snRNP assembly, analogous to nucleoli in ribosome assembly³⁶. The relationship between these snRNP-containing spheres and the structures that give rise to the speckled staining pattern in somatic cells is still unclear.

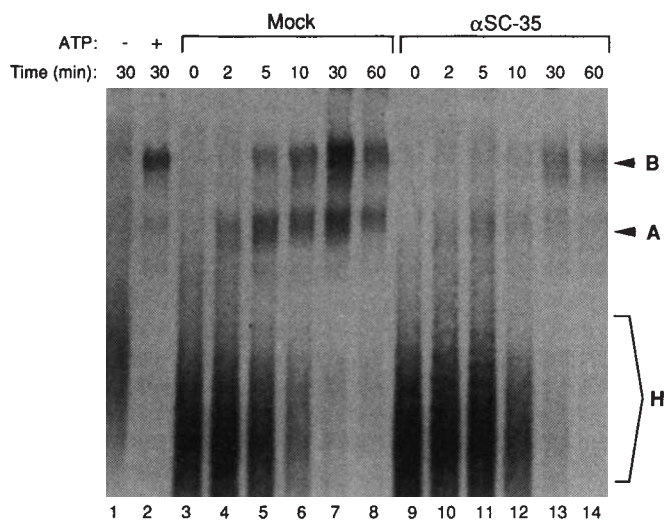
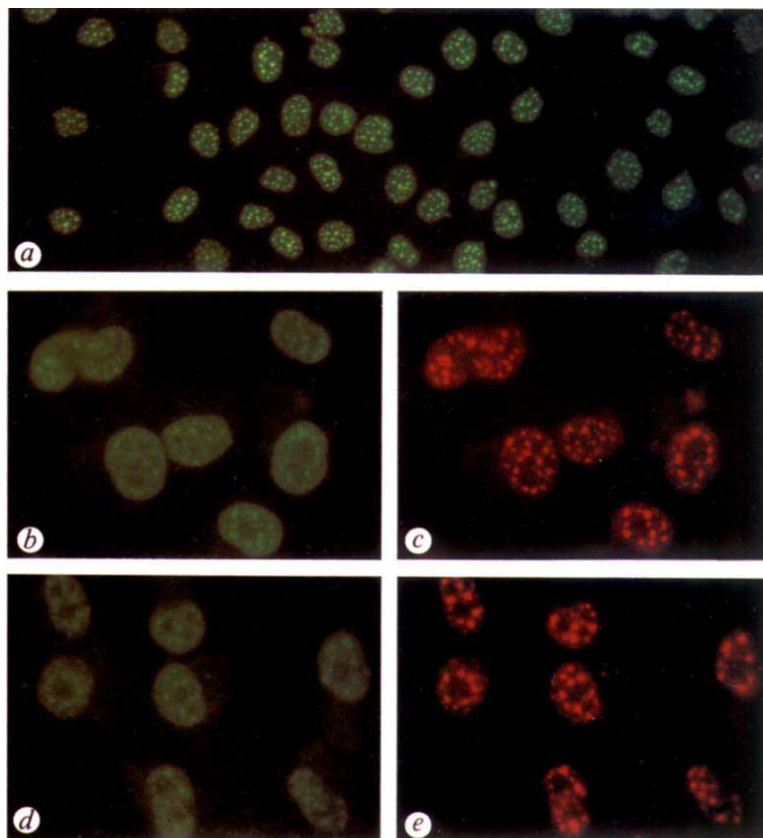


FIG. 5 The SC-35 antigen is required for both A and B complex formation. Mock-depleted (lane 3–8) and SC-35-depleted (lane 9–14) nuclear extracts were incubated with ³²P-labelled Ad10 plasmid RNA (Ad10 was a gift from M. Garcia-Blanco, M. Konarska and P. Sharp) under splicing conditions for the times indicated. Splicing complexes were fractionated by electrophoresis on a 4% polyacrylamide Tris–glycine-buffered gel²⁷. Migration of complexes B, A, and H are indicated on the right. Complex formation in untreated nuclear extracts in the absence (lane 1) or presence (lane 2) of ATP is shown.

FIG. 6 Nuclear localization of the SC-35 antigen. *a*, Indirect immunofluorescent staining of human MG63 osteosarcoma cells using the α SC-35 mAb. *b* and *c*, Double immunofluorescent staining of human MG63 cells with human α Sm serum (*b*) and mouse α SC-35 mAb (*c*). *d* and *e*, Double immunofluorescent staining with human α U1 serum (*d*) and mouse α SC-35 mAb (*e*) (α Sm and α U1 human sera were gifts from J. Bruzik and J. A. Steitz). The human and mouse antibodies were probed by anti-human immunoglobulin-fluorescein conjugator (*b* and *d*) and anti-mouse immunoglobulin-rhodamine conjugator (*c* and *e*) respectively.

METHODS. MG63 cells were grown overnight on slides (Flow Labs), fixed in 2% formaldehyde/PBS/0.2% Triton X-100 for 10 min at 4 °C and permeabilized for 5 min in cold acetone (−20 °C). Treated cells were stored in PBS at 4 °C for up to one week. For indirect immunofluorescent microscopy, fixed cells were blocked for 30 min in 20% fetal bovine serum/PBS/0.5% Tween 20. Antibodies (10 μ l of culture supernatant, 1 in 2,000 dilution of sera, or 1 to 50 μ g ml^{−1} purified mAbs) in blocking solution were applied to the slides and incubated for 1 to 2 h at room temperature. Slides were washed for 10 min in PBS and treated for 1 h with the secondary fluorescein- or rhodamine-labelled anti-mouse or anti-human IgG (1:100 dilution in blocking buffer; Cappel), washed three times in PBS and mounted.



Immunoelectron microscopy shows that most of the speckled regions enriched with snRNPs are not connected to the nuclear envelope^{17,37,38}. Rather, they appear to form a reticular network within the nucleoplasm, which extends from the centre of the nucleus to the nuclear membrane³⁷. Optical sectioning of cells stained with α SC-35 antibody with a confocal microscope reveals speckled regions distributed throughout the nucleoplasm and not concentrated near the nuclear membrane (data not shown). This intranuclear distribution differs from that of the yeast splicing factor, *RNA11*, and yeast snRNPs, both of which are localized in a diffuse pattern near the nuclear membrane³⁹;

we do not know whether or not this difference in the localization of splicing components in yeast and higher eukaryotes reflects a fundamental difference in the mechanism of pre-mRNA splicing and mRNA transport.

In summary, we have shown that a non-snRNP component of mammalian spliceosomes is concentrated in some 20–50 discrete regions within the nucleus. These regions are also enriched in the snRNPs that are required for spliceosome assembly and pre-mRNA splicing. Thus, the processes of pre-mRNA splicing or spliceosome assembly, or both, are compartmentalized in mammalian nuclei. □

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1. Krainer, A. R. & Maniatis, T. In *Frontiers in Transcription and Splicing* (ed. Hames, B. D. & Glover, D. M.) 131–206 (IRL, Oxford and Washington, 1988).
2. Steitz, J. A. *et al.* In *Structure and Function of Major and Minor Snurps* (ed. Birnstiel, M. L.) 115–154 (Springer, Heidelberg, 1988).
3. Krainer, A. R. & Maniatis, T. *Cell* **42**, 725–736 (1985).
4. Kramer, A. & Keller, W. *EMBO J.* **4**, 3571–3581 (1985).
5. Furneaux, H. M., Perkins, K. K., Freyer, G. A., Arenas, J. & Hurwitz, J. *Proc. natn. Acad. Sci. U.S.A.* **82**, 4351–4355 (1985).
6. Perkins, K. K., Furneaux, H. M. & Hurwitz, J. *Proc. natn. Acad. Sci. U.S.A.* **83**, 887–891 (1986).
7. Kramer, A., Frick, M. & Keller, W. *J. biol. Chem.* **262**, 17630–17640 (1987).
8. Ruskin, B., Zamore, P. D. & Green, M. R. *Cell* **52**, 207–219 (1988).
9. Gerke, V. & Steitz, J. A. *Cell* **47**, 973–984 (1986).
10. Tazi, J. *et al.* *Cell* **47**, 755–766 (1986).
11. Swanson, M. S. & Dreyfuss, G. *EMBO J.* **7**, 3519–3529 (1988).
12. Garcia-Blanco, M. A., Jamison, S. R. & Sharp, P. A. *Genes Dev.* **3**, 1874–1886 (1989).
13. Reed, R., Griffith, J. & Maniatis, T. *Cell* **53**, 949–961 (1988).
14. Abmayr, S. M., Reed, R. & Maniatis, T. *Proc. natn. Acad. Sci. U.S.A.* **85**, 7216–7220 (1988).
15. Lerner, E. A., Lerner, M. R., Janeway, C. A. & Steitz, J. A. *Proc. natn. Acad. Sci. U.S.A.* **78**, 2737–2741 (1981).
16. Deng, J. S., Takasaki, Y. & Tan, E. M. *J. Cell Biol.* **91**, 572–577 (1981).
17. Spector, D. L., Schrier, W. H. & Busch, H. *Biol. Cell* **49**, 1–10 (1983).
18. Reuter, R., Appel, B., Bringmann, P., Rinke, J. & Luhrmann, R. *Expl Cell Res.* **154**, 548–560 (1984).
19. Nyman, U., Hallman, H., Hadjilacy, I., Sharp, G. & Ringertz, N. R. *J. Cell Biol.* **102**, 137–144 (1986).
20. Chabot, B., Black, D. L., LeMaster, D. M. & Steitz, J. A. *Science* **230**, 1344–1349 (1985).
21. Krainer, A. R. *Nucleic Acids Res.* **16**, 9415–9429 (1988).
22. Hinterberger, M., Pettersson, I. & Steitz, J. A. *J. biol. Chem.* **258**, 2604–2613 (1983).

23. Kinlaw, C. S., Roberson, B. L. & Berget, S. M. *J. biol. Chem.* **258**, 7181–7189 (1983).
24. Grabowski, P. J., Seiler, S. R. & Sharp, P. A. *Cell* **42**, 345–353 (1985).
25. Frendewey, D. & Keller, W. *Cell* **42**, 355–367 (1985).
26. Konarska, M. M. & Sharp, P. A. *Cell* **46**, 845–855 (1986).
27. Konarska, M. M. & Sharp, P. A. *Cell* **49**, 763–774 (1987).
28. Bindereif, A. & Green, M. R. *EMBO J.* **6**, 2415–2424 (1987).
29. Kramer, A. *Genes Dev.* **2**, 1155–1167 (1988).
30. Mariman, E. C. M., van Eekelen, C. A. G., Reinders, R. J., Berns, A. J. M. & van Venrooij, W. J. *J. molec. Biol.* **154**, 103–119 (1982).
31. Ciejek, E. M., Nordstrom, J. L., Tsai, M.-J. & O'Malley, B. W. *Biochemistry* **21**, 4945–4953 (1982).
32. Spector, D. L. *Biol. Cell* **51**, 109–112 (1984).
33. Spector, D. L., Watt, R. A. & Sullivan, N. F. *Oncogene* **1**, 5–12 (1987).
34. Osheim, Y. N., Miller, O. L. & Beyer, A. L. *Cell* **43**, 143–151 (1985).
35. Beyer, A. L. & Osheim, Y. N. *Genes Dev.* **2**, 754–765 (1988).
36. Gall, J. G. & Callan, H. G. *Proc. natn. Acad. Sci. U.S.A.* **86**, 6635–6639 (1989).
37. Spector, D. L. *Proc. natn. Acad. Sci. U.S.A.* (in the press).
38. Fakan, S., Leser, G. & Martin, T. E. *J. Cell Biol.* **98**, 358–363 (1984).
39. Chang, T.-H., Clark, M. W., Lustig, A. J., Cusick, M. E. & Abelson, J. *Molec. cell. Biol.* **8**, 2379–2393 (1988).
40. Bradford, M. M. *Analyt. Biochem.* **72**, 248–254 (1976).
41. Taggart, R. T. & Samloff, I. M. *Science* **219**, 1228–1230 (1983).
42. Kiehart, D., Kaiser, D. A. & Pollard, T. D. *J. Cell Biol.* **99**, 1002–1014 (1984).

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Upper limit set for level of lightning activity on Titan

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LIGHTNING is known to occur in the atmospheres of Earth and Jupiter¹, and there is strong evidence of lightning on Saturn² and Uranus³. Based on its extensive atmosphere, the presence of aerosols and the deposition of significant amounts of solar energy at its surface, it has been calculated that Saturn's largest moon, Titan, may produce lightning with an energy dissipation rate somewhat less than that at Earth⁴. An opportunity to search for evidence of lightning at Titan occurred during the Voyager 1 encounter with Saturn on 12 November 1980, when the spacecraft passed within 4,394 km of Titan's cloud tops. Because optically thick cloud and haze layers prevented lightning detection at optical wavelengths, we have searched for lightning-radiated signals (spherics) at radio wavelengths using the planetary radioastronomy instrument⁵ aboard Voyager 1. Given the maximum ionosphere density^{6,7} of $\sim 3 \times 10^3 \text{ cm}^{-3}$, lightning spherics should be detectable above an observing frequency of 500 kHz. Failing to find any evidence for lightning-associated spherics, we infer an upper limit to the total energy per flash in Titan lightning of $\sim 10^6 \text{ J}$, or about a thousand times weaker than that typical of terrestrial lightning. The level of lightning activity on Titan has implications for the production of certain hydrocarbons in its atmosphere and for the design of instruments on spacecraft such as Cassini, which is scheduled to arrive at Saturn in 2002.

Figure 1 shows the Voyager 1 trajectory past Titan with closest approach occurring at about 05:40 UT when the spacecraft was $2.72 R_T$ (the radius of Titan, R_T , is 2,575 km) from the centre of the satellite. To allow sufficient time to record possible rare events but also recognizing the need for highly sensitive measurements, we restricted the observations to an interval within $\pm 50 \text{ min}$ of closest approach. During this interval Voyager 1 was with $17 R_T$ of Titan, corresponding to a range of 10^2 ($17/2.72 - 1$)² in sensitivity. About 98% of Titan's surface was 'visible' to the spacecraft during this interval.

Figure 2 (top) illustrates the characteristic signatures of what are believed to be lightning spherics from Saturn² as they appeared in a Voyager planetary radioastronomy instrument (PRA) spectrogram taken on 13 November 1980. The spacecraft was about four planetary radii from Saturn, above the nightside,

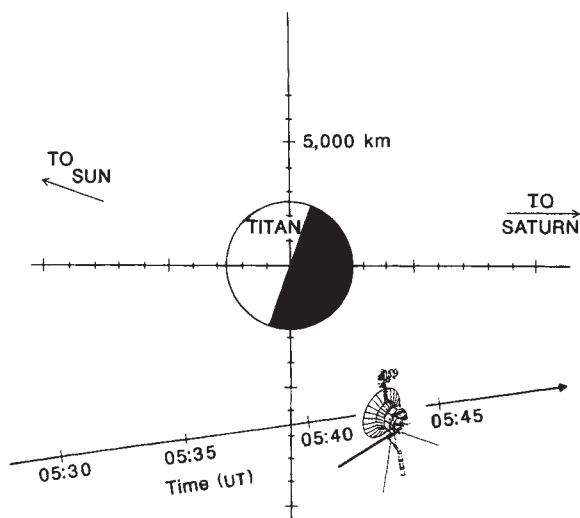


FIG. 1 Trajectory of Voyager 1 past Titan (12 November 1980) projected into the equatorial plane of Saturn.

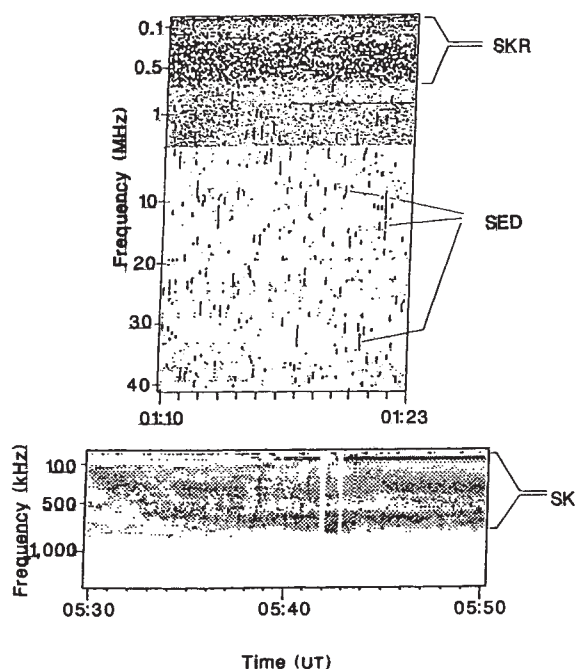


FIG. 2 The top panel shows a radio spectrogram of lightning-like discharges from Saturn on 13 November 1980. The signals (Saturn electrostatic discharge, SED) appear as short vertical streaks because the swept-frequency operation of the PRA instrument causes the emission to be detected only in those channels the receiver happens to be sampling during the short-lived duration of the flash. In the bottom panel, a radio spectrogram of the 20-min interval centred on Voyager closest approach to Titan (marked by the arrow) shows only the diffuse band of Saturn kilometric radiation (SKR). No discharge-like phenomenon is seen. The interval shown corresponds to the portion of the trajectory shown in Fig. 1. The higher frequency portion of the PRA receiver band (1.3–40 MHz) is not included because of excessive noise.

when these signals were observed. The emissions appear as short vertical streaks scattered randomly in frequency and time. We examined radio spectrograms and also single-channel records for similar lightning-like signatures during the Titan flyby period. During the entire 100-min period of the Titan flyby, only kilometre-wavelength radiation originating in Saturn's auroral regions⁸ (called Saturn kilometric radiation, SKR) was detected. The SKR was confined to frequencies below $\sim 750 \text{ kHz}$ and is visible in Fig. 2 (bottom) as a diffuse band of emission. Above $\sim 500 \text{ kHz}$, where Titan's ionosphere becomes transparent, and in particular above $\sim 750 \text{ kHz}$, where the auroral (SKR) noise level is extremely low, we expect to see Titan spherics if they exist. However, no signals were detected in excess of the effective PRA receiver threshold of $\sim 2 \times 10^{-20} \text{ W m}^{-2} \text{ Hz}^{-1}$, corresponding to a threshold signal strength of $0.10 \mu\text{V m}^{-1}$ over the PRA bandwidth of 1 kHz per channel.

Comparison of this observing threshold with the expected radiated power from terrestrial-like lightning is instructive. Each flash of terrestrial lightning radiates an energy, W_t , of $\sim 10^4 \text{ J}$ at radio wavelengths^{9,10}. The peak flux occurs near 10 kHz with a spectrum that falls off as the inverse square of the frequency f at the high-frequency end^{11,12}. Near 500 kHz the spectral energy W_f ($W_t = \int W_f df$) is $\sim 4 \times 10^{-4} \text{ J Hz}^{-1}$. At the distance of Voyager 1 from Titan, d , the corresponding field strength E is $\sim 5 \mu\text{V m}^{-1}$, given by $E^2 = Z_0 W_f \Delta f / 4\pi d^2 \Delta t$, where Z_0 is the impedance of free space (120π), Δf and Δt are the PRA receiver passband (1 kHz) and integration time (25 ms) and d is 4,400 km. By comparison, direct satellite measurement of terrestrial lightning at radio frequencies¹² yields $E \sim 50 \mu\text{V m}^{-1}$ near 500 kHz. Thus the typical range of signal strengths expected at the spacecraft is $5 \leq E \leq 50 \mu\text{V m}^{-1}$, corresponding to a factor $\sim 10^3$ – 10^5 in energy above the PRA receiver threshold of

$0.10 \mu\text{V m}^{-1}$. Therefore at the distance of the Titan flyby, lightning would be detectable at a level three to five orders of magnitude weaker than that typical of terrestrial lightning.

Could Titan lightning exist but effectively avoids detection owing to special circumstances? For example, if the spectral peak of the lightning is at $\ll 10$ kHz, the flux at $f \geq 500$ kHz would be reduced, all else being equal. The location of the spectral peak is determined by the Fourier transform of the pulse waveform¹³. A lower-frequency spectral peak would require a longer lightning discharge channel length, possibly a few orders of magnitude longer than the 3–9-km channels seen at Earth. If this unlikely possibility were the case then, because the radiated electrical power scales as the fourth power of the charge separation¹⁴, the resultant radio-emission level would be boosted by an amount at least commensurate with the power loss due to the spectral peak shift.

We also considered the possibility that the radiofrequency power spectrum falls off faster than f^{-2} , which would yield less radio power at $f \geq 500$ kHz. The factors controlling the spectral shape are the discharge-path orientation and its 'tortuosity'. Models¹³ in which these factors were varied yielded spectral indexes between -1 and -2 , indicating that our analysis actually represents a lower limit to the expected power at high frequencies.

Finally, we considered the importance of signal absorption along the path to the spacecraft. Measurements of Titan's atmosphere¹⁵ and high-altitude ionosphere⁶, derived from the Voyager 1 ultraviolet-spectrometer experiment, and models of Titan's low-altitude cosmic-ray induced ionosphere¹⁶ were used to estimate the total attenuation. Although lightning discharges would probably be generated in the troposphere below ~ 35 km altitude⁴, nearly all of the absorption takes places in the ionosphere layers near 100 and 1,000 km altitude. Here the peak electron densities are $\sim 1,000$ and $\sim 3,000 \text{ cm}^{-3}$, and the nitrogen

molecule densities are $\sim 5 \times 10^{18}$ and $\sim 10^{10} \text{ cm}^{-3}$, respectively. Under these circumstances, the signal absorption is dominated by collisions between electrons and neutral molecules^{17,18}. We find that, over the extent of the two ionospheres, the attenuation of a 750-kHz lightning signal will be < 0.50 dB, corresponding to $> 89\%$ transmission.

We have shown that if Earth-like lightning existed on Titan it would have been easily detected by PRA during the Voyager flyby. On the other hand, the predicted⁴ energy dissipation rate of Titan lightning ($4 \times 10^{-6 \pm 1} \text{ W m}^{-2}$) is less than that typically seen at earth ($8 \times 10^{-5} \text{ W m}^{-2}$, ref. 19). Could this reduced level of emission have been detected? We plot the energy per flash E_{fl} as a function of the number N_{fl} of flashes per unit area and time (Fig. 3) to show the parameter regime that is effectively excluded by the observations. As shown by the shaded area, the excluded regions cover Earth-like lightning and a large portion of the area predicted for Titan (stippled swath). The region of the predicted area in the lower right corner cannot be excluded on the basis of the present observations, however, because E_{fl} here is below the PRA detection threshold. This region has $E_{\text{fl}} \leq 10^6 \text{ J}$ and $N_{\text{fl}} \geq \sim 100$. The latter is equivalent globally to $\geq \sim 200$ flashes s^{-1} . For terrestrial lightning, $E_{\text{fl}} \sim 10^9 \text{ J}$ and $N_{\text{fl}} \sim 100$ flashes s^{-1} . Therefore, if the energy dissipation rate on Titan is comparable to that predicted⁴, our observations indicate that the total energy per flash is $\sim 10^3$ times lower than that typical at Earth, whereas the flash rate must be comparable or much higher. Although this seems to be an unusual parameter regime in which to find lightning discharges, we stress that it cannot be dismissed out of hand, because the physics of lightning is poorly understood. The observations do, however, clearly rule out the presence of terrestrial-like discharges.

The presence or absence of lightning activity on Titan has important consequences for the inventory of hydrocarbons in Titan's atmosphere. For example, the observed abundances of hydrogen cyanide, acetylene, ethane and propane are in agreement with the calculated abundances derived photochemically²⁰. The model prediction for ethylene falls short of the observations by an order of magnitude, however, and cannot be adequately accounted for except by a significant level of lightning activity²¹. As the lightning-discharge level is probably very low, our results indicate that either another process is involved or that certain aspects of the chemical kinetics of organic synthesis in Titan's atmosphere should be re-evaluated.

Many questions concerning Titan, and the Saturn system in general, will be answered by the proposed NASA/ESA Cassini mission, which is scheduled to arrive at Saturn in the year 2002. Cassini will consist of a Titan probe and Saturn orbiter. Model payloads on both the probe and orbiter contain experiments that should definitely settle the question of the existence of lightning on Titan provided adequate attention is paid to issues of instrument sensitivity and spacecraft-generated interference. Under ideal conditions, the orbiter radio-emissions detector should realize about a factor of 100 improved detection capability relative to the Voyager 1 results presented here. Although the Titan-probe instrument will have even better detection capability than the orbiter because of its ability to make observations both near the spectral peak and near the source of any lightning that may exist, the observations will effectively be limited to altitudes below 100 km, where only a very small fraction of the satellite is 'visible'. We conclude, therefore, that neither the probe nor the orbiter instrument by itself should be relied on to make the definitive search for Titan lightning. $\square$

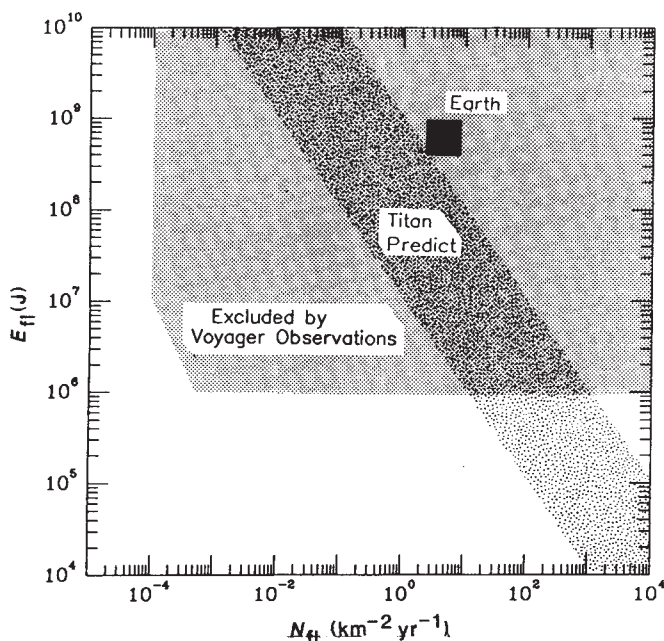


FIG. 3 This shows the region that can be excluded by the Voyager 1 observations plotted in terms of the energy per lightning flash E_{fl} (J) and flash rate N_{fl} ($\text{km}^{-2} \text{ yr}^{-1}$). The product of $E_{\text{fl}} N_{\text{fl}}$, in the proper units, yields the global energy dissipation rate (W m^{-2}), a commonly used unit describing lightning output. We assume a flash duration of 25 ms and a ratio of radio-to-total radiated power of 5×10^{-5} . The slanted limit to the lower left of the figure is due to the changing threshold during the 100-min analysis interval. The energy and flash rate of terrestrial lightning are shown. The corresponding values for Jupiter and Saturn are not well known; however, they are probably somewhat greater than the Earth in terms of energy per flash²².

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1. Smith, B. A. *et al. Science* **204**, 951–972 (1979).
2. Kaiser, M. L., Connerney, J. E. P. & Desch, M. D. *Nature* **303**, 50–53 (1983).
3. Zarka, P. & Pedersen, B. M. *Nature* **323**, 605–608 (1986).
4. Borucki, W. J., McKay, C. P. & Whitten, R. C. *Icarus* **60**, 260–273 (1984).
5. Warwick, J. W. *et al. Space Sci. Rev.* **21**, 309–319 (1977).
6. Strobel, D. F. & Shemansky, D. E. *J. geophys. Res.* **87**, 1361–1368 (1982).
7. Lindal, G. F. *et al. Icarus* **53**, 348–363 (1983).

8. Kaiser, M. L., Desch, M. D. & Lecacheux, A. *Nature* **292**, 731–733 (1981).
9. Horner, F. *Advances in Radio Research* Vol. 2 (ed. Saxon, J. A.) 121–204 (Academic, New York, 1964).
10. Taylor, W. L. *J. Res. natn. Bur. Stand.* **67D**, 539–550 (1963).
11. Lanzerotti, L. J. et al. *J. geophys. Res.* **94**, 13221–13227 (1989).
12. Kotaki, M. & Katoh, C. *J. atmos. terr. Phys.* **45**, 833–847 (1983).
13. LeVine, D. M. & Meneghini, R. *Radio Sci.* **13**, 801–809 (1978).
14. Vonnegut, B. *Met. Monogr.* **5**, 224–241 (1963).
15. Smith, G. R. et al. *J. geophys. Res.* **87**, 1351–1359 (1982).
16. Borucki, W. J. et al. *Icarus* **72**, 604–622 (1987).
17. Ratcliffe, J. A. *The Magnetoionic Theory and its Applications to the Ionosphere* (Cambridge University Press, 1962).
18. Zarka, P. *Astr. Astrophys.* **146**, L15–L18 (1985).
19. Borucki, W. J. & Chameides, W. L. *Rev. Geophys. Space Phys.* **22**, 363–372 (1984).
20. Yung, Y. L., Allen, M. & Pinto, J. P. *Astrophys. J. Suppl. Ser.* **55**, 465–506 (1984).
21. Borucki, W. J. et al. *Icarus* **76**, 125–134 (1988).
22. Rinnert, K. *J. geophys. Res.* **90**, 6225–6237 (1985).

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Direct evidence for a very large penetration depth in superconducting $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$ single crystals

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THE temperature-dependent magnetic penetration depth $\lambda(T)$ and the superconducting coherence length $\xi(T)$ are the two fundamental lengths in superconductivity¹, and it is clearly important to determine these two quantities in the new high-transition-temperature (high- T_c) oxide superconductors². Here we report a direct determination of $\lambda(0)$ for high-quality single crystals of $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$ with a T_c of 85 K, for the particular case in which the screening currents responsible for the diamagnetism flow perpendicular to the CuO_2 planes. The measured value, $\lambda_c(0) = 100 \pm 10 \mu\text{m}$, is surprisingly large, but it is consistent with the anisotropy in the electrical resistivity above T_c , measured on the same batch of single crystals, and approximately consistent with the anisotropy in lower critical field, H_{c1} , measured by others³. Such a large value may have important consequences for the physical properties of polycrystalline samples and thin films.

Although there is as yet no consensus on which theory correctly describes the new high- T_c oxide superconductors, the order of magnitude of the magnetic penetration depth is expected to be given by the London formula⁴

$$\lambda_L(0) = \sqrt{\frac{m^* c^2}{4\pi n e^2}} \quad (1)$$

where n is the density and m^* the effective mass of the carriers responsible for superconductivity, e is the electronic charge and c the velocity of light, in c.g.s. units. In oxide superconductors the anisotropy in m^* is therefore expected to give substantial anisotropy in λ . The smaller value λ_{ab} refers to the case where the screening currents flow in the highly conducting CuO_2 or ab planes and the larger value λ_c is relevant when the screening currents flow in the low-conductivity direction perpendicular to the CuO_2 planes^{2,5}.

Various experimental methods have been used to determine $\lambda(0)$ and $\lambda(T)$ for oxide superconductors. A conceptually simple and direct method is to measure the diamagnetism of small particles of different sizes^{6–8} or of small single crystals^{9,10} and we use the latter method here. Although they are still controver-

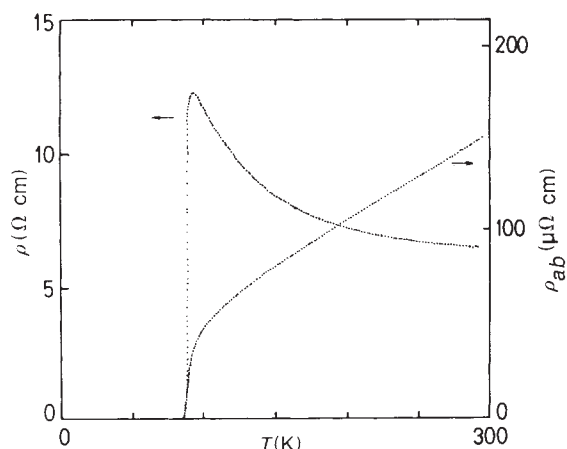


FIG. 1 Resistivity-temperature curves for current flow parallel (ρ_{ab}) and perpendicular (ρ_c) to the CuO_2 layers of $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$.

sial we note that we have no reason to doubt the validity of the results in ref. 6.

Alternatively, λ can be determined from measurements in the vortex state above H_{c1} (especially from muon spin relaxation^{11–13}, from H_{c1} itself⁵ or from the logarithmic slope of the magnetization curves¹⁴. All of these measurements depend on the use of Ginzburg–Landau theory to determine λ , whereas the direct measurements may be oversensitive to the surface properties of the particles or crystals. In the direct method used here, however, the influence of surface defects is reduced by cleaving the crystal into successively smaller pieces. The technique used here is very simple and can easily be extended to determine $\lambda_c(T)$ for these crystals, and for other crystals or oriented films of oxide superconductors.

We prepared $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$ (2212) crystals by cooling a melt, as described elsewhere¹⁵. The crystals were extensively characterized using electrical measurements such as resistivity anisotropy (Fig. 1, for example) and the Hall effect^{16,17}. Photographs made with an X-ray Weissenberg camera (O. Milat, personal communication) showed the same crystal structure as in refs 18 and 19 and a mosaic spread of 1° .

Figure 1 shows typical in-plane, $\rho_{ab}(T)$, and out-of-plane, $\rho_c(T)$, resistivities for our 2212 crystals. $\rho_{ab}(T)$ agrees well with previous reports¹⁹ and extrapolation gives a low residual value at $T = 0$, which is probably an indication of a high degree of order in the CuO_2 planes. By contrast, $\rho_c(T)$ shows non-metallic behaviour, increasing by a factor of two from room temperature down to 90 K. The anisotropy ratio ρ_c/ρ_{ab} is 4.2×10^4 at 300 K and $\sim 3 \times 10^5$ at 100 K.

Our main results, the a.c. susceptibility data for a thin $1 \times 0.5 \times 0.017 \text{ mm}^3$ 2212 crystal with measuring field parallel to the CuO_2 layers, are shown in Fig. 2. Experiments on similar crystals showed that the data were not influenced by the amplitude of the a.c. field ($< 0.9 \text{ G}$, r.m.s.), its frequency (332 Hz) or by the presence of the Earth's magnetic field. We determined the volume of the crystal and hence the a.c. signal corresponding to perfect diamagnetism, from its weight ($47 \mu\text{g}$) and the X-ray density of 6.5 g cm^{-3} . The crystal was cleaved lengthwise under a microscope using a razor blade, into two and then into four approximately equal pieces. As shown in Fig. 2, the diamagnetism was reduced systematically from the initial value of 52% to 36% and then to 17% by cutting. When we rotated the four pieces by 90° (keeping the a.c. field in the ab plane) the diamagnetism returned to its original value, as shown in the upper part of Fig. 2. This proved that the crystal was not damaged by cutting, for example, it did not split into thinner layers and there was no significant weight loss. Similar results were obtained for another crystal for which we had verified, before cutting, that turning the crystal by 90° did not alter the diamagnetic signal.

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TABLE 1 $T=0$ penetration depths

Crystal	$\chi(T=0)/\chi_{\max}$	W (μm)	x ($\equiv 2\lambda_c/W$)	$\lambda_c(0)$ (μm)
2212-KB14	0.55	500	0.48	120
	0.34	250	0.78	98
	0.115	125	1.55	97
2212-KB15	0.52	590	0.5	148
	0.36	295	0.72	106
	0.17	148	1.3	96

Because the crystals were very thin and the pieces separated by ≥ 0.1 mm when they were placed side by side on the quartz sample holder, demagnetization effects and magnetic interactions between pieces were negligible. Therefore, even without detailed analysis, the most probable interpretation of the loss of diamagnetism is that the widths ($W \sim 100 \mu\text{m}$) of the cut crystals are of the order of λ_c . From muon-spin-relaxation work¹³ it is known that $\lambda_{ab}(0)$ of 2212 is approximately the same as for $\text{YBa}_2\text{Cu}_3\text{O}_7$ crystals, that is, $\lambda_{ab}(0) = 140$ nm (refs 11, 12). From the diagram of the crystal cross-section in Fig. 3a, it can be seen that for $\lambda_c \sim W$ ($\approx 100 \mu\text{m}$) and λ_{ab} (~ 140 nm) $\ll t$ ($17 \mu\text{m}$), most of the field penetration occurs along the width of the crystal. Using a coordinate transformation²⁰, it can be shown that the diamagnetism χ is given by the thin-plate formula— $\chi/\chi_{\max} = 1 - x \tanh(1/x)$ (ref. 4) with $x = 2\lambda_c/W$. We have used this formula to estimate $\lambda_c(0)$ from the data in Fig. 2 and for another crystal, and the results are summarized in Table 1. After cutting, the values obtained for λ_c are consistently equal to $100 \pm 10 \mu\text{m}$. We believe that the somewhat larger values obtained for the uncut crystals may be attributed to the effects of steps or other irregularities of the ab -plane crystal surface. As sketched in Fig. 3b, steps in the ab plane, which occur for most crystal surfaces, could easily reduce the diamagnetism because of the large value of λ_c . Only a 20% reduction in diamagnetism (or a 10% aspect ratio for the steps) for the uncut crystals would bring the values of λ_c into line with those for the cut crystals.

We note that we are in the opposite limit from that relevant for the $\text{YBa}_2\text{Cu}_3\text{O}_7$ crystal used in ref. 10. 2212 crystals have a much lower c -axis conductivity and a much larger value of λ_c , so that $\lambda_c/W > \lambda_{ab}/t$ and the diamagnetism is determined by the ratio λ_c/W . By contrast, the diamagnetism in ref. 10 was determined by λ_{ab}/t , which was larger than λ_c/W , and therefore reducing W by cutting the crystal lengthwise should have had no effect on the diamagnetism.

In a simple Drude model the electrical resistivity above T_c is given by $\rho = m^*/ne^2\tau$, where τ is the scattering time, and hence the anisotropy in the penetration depth should be equal to the square root of the resistivity anisotropy. Our value of $\lambda_c(0)$ and the published value of $\lambda_{ab}(0) = 140$ nm (refs 11, 12) give $\lambda_c/\lambda_{ab} = 710$ in agreement with $\sqrt{\rho_c/\rho_{ab}} = 550$ at 100 K (Fig. 1). (We consider agreement to within a factor of two satisfactory because the unusual non-metallic temperature dependence of ρ_c is not understood.) If H_{c1} of the 2212 crystals for fields perpendicular to the CuO_2 layers is assumed to be the same as for $\text{YBa}_2\text{Cu}_3\text{O}_7$ crystals, 690 G (ref. 5), which should be so if the λ_{ab} values are the same, then, because of the large anisotropy in λ , the in-plane value of H_{c1} is only 1 G. This is reasonably close to the published value³ ≈ 4 G.

We have also shown²¹ that the oxygen content of 2212 crystals can be altered reversibly, reducing T_c continuously from 85 to 40 K, and at the same time even larger values of $\lambda_c(0)$, up to 500 μm , are obtained.

Our interpretation of $\lambda_c(0)$ as a London penetration depth for an extremely large value for the effective mass along the c direction is not the only possibility. The large value of λ_c could arise from weak Josephson coupling between the superconducting planes and equation (1) would not then be applicable. In

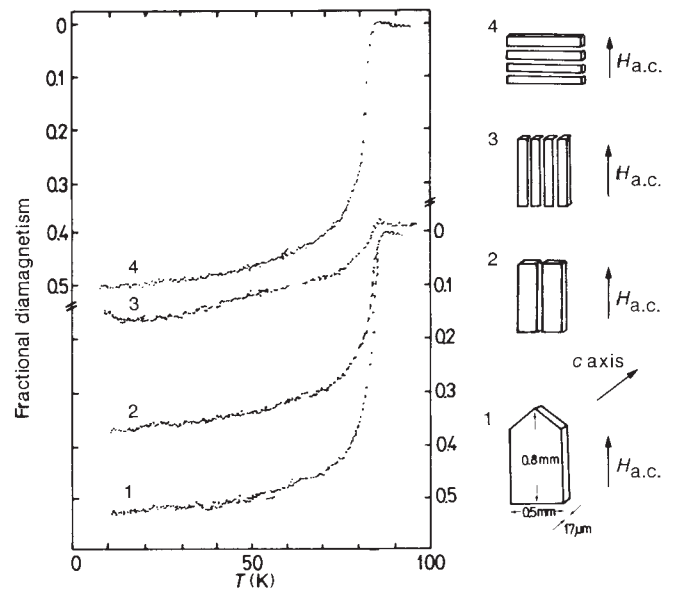


FIG. 2 A.c. susceptibility data for a single crystal of 2212, before (curve 1) and after (curves 2 and 3) cutting it lengthwise in the ab plane. When the four pieces of the cut crystal were rotated by 90° (curve 4) the original diamagnetic signal was restored. In all cases the a.c. field was in the ab plane, and the c axis was perpendicular to the plane of the diagram.

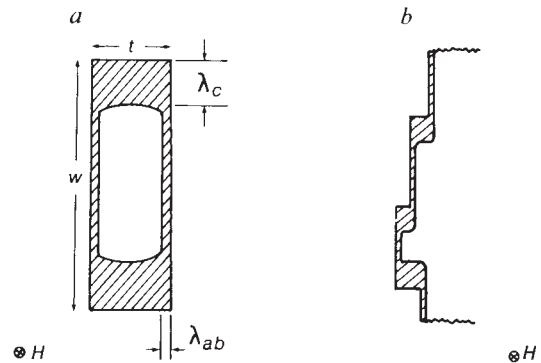


FIG. 3 a, Sketch of crystal cross-section (not to scale)—shaded area shows region of field penetration (that is, current flow) in the limit $\lambda_c \sim W$ and $\lambda_{ab} \ll t$. b, Steps in the ab surface increase field penetration in the c direction. In both diagrams the c axis is horizontal and in the plane of the paper, and the a.c. field is perpendicular to c .

that case there would presumably be substantial differences between the values of λ_c deduced from direct measurements and those derived from the properties of the vortex state, or from the value of H_{c1} .

In principle, the data in Fig. 2 can also be used to obtain $\lambda_c(T)$. In many of our measurements on 2212 crystals with $T_c = 85$ K, there is a small T^2 term in the diamagnetism at low temperatures, which probably corresponds to a T^2 term in the penetration depth similar to that found from measurements on $\text{YBa}_2\text{Cu}_3\text{O}_7$ powders⁶. This point is controversial, however, because many groups have claimed that their data is consistent with normal s-wave superconductivity. More detailed experiments and analysis are therefore under way so that we may clarify the temperature dependence of λ_c . □

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1. Tinkham, M. *Introduction to Superconductivity* (McGraw-Hill, New York, 1975).
2. Forgan, E. M. *Nature* **329**, 483–485 (1987).
3. Liu, J. J. *et al. Phys. Rev. B* **38**, 5095–5097 (1988).
4. Shoenberg, D. *Superconductivity* (Cambridge University Press, 1960).
5. Umezawa, A. *et al. Phys. Rev. B* **38**, 2843–2864 (1988).
6. Cooper, J. R., Chu, C. T., Zhou, L.-W., Dunn, B. & Grüner, G. *Phys. Rev. B* **37**, 638–641 (1988).
7. Ishida, T. & Mazaki, H. *J. appl. Phys.* **26**, L2003–2006 (1987).

8. Monod, P., Dubois, B. & Odier, P. *Physica C* **153-155**, 1489-1490 (1988).
9. Cooper, J. R. *et al. Physica C* **153-155**, 1491-1492 (1988).
10. Krusin-Elbaum, L., Greene, R. L., Holtzberg, F., Malozemoff, A. P. & Yeshurun, Y. *Phys. Rev. Lett.* **62**, 217-220 (1989).
11. Uemura, Y. J. *et al. Phys. Rev.* **B38**, 909-912 (1988).
12. Harshmann, D. R. *et al. Phys. Rev.* **B39**, 851-854 (1988).
13. Uemura, Y. J. *et al. Phys. Rev. Lett.* **62**, 2317-2320 (1989).
14. Kogan, V. G., Fang, M. M. & Mitra, S. *Phys. Rev.* **B38**, 11958-11961 (1988).
15. Keszei, B., Szabo, Gy., Vandek, J., Pogany, L. & Oszlany, G. *J. Less Common Metals* (submitted).
16. Forró, L. & Cooper, J. R. *Europhysics Lett.* (in the press).
17. Forró, L. & Hamzić, A. *Solid St. Commun.* (in the press).
18. Takagi, H. *et al. Nature* **332**, 236-238 (1988).
19. Sunshine, S. A. *et al. Phys. Rev.* **B38**, 893-896 (1988).
20. Fraser, A. R. & Shoenberg, D. *Proc. Camb. phil. Soc. math. phys. Sci.* **45**, 680-683 (1949).
21. Forró, L., Cooper, J. R., Leontić, B. & Keszei, B. *Europhysics Lett.* **10**, 371-374 (1989).

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Guest-responsive structural changes in cholic acid intercalation crystals

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CHOLIC acid forms multimolecular inclusion compounds with a variety of organic substances¹. Some of these inclusion compounds have crystal structures containing channels², which can perform efficient chiral recognition of, for example, lactones³; others, with ethanol⁴ or water⁵ as guest molecules for example, have no such channels. Although these two types of structure seem at first to be very different, we report here results that indicate that they are closely related. In particular we present the novel finding that guests can be added, removed or exchanged without the intervention of an amorphous state. We suggest that cholic acid crystals have a dynamic layered structure which can be modified to incorporate guests by rearrangement of the intermolecular hydrogen-bonded networks. To our knowledge, this is the first example of intercalation in organic crystals accompanied by guest-responsive on-off control of channels, although many similar inorganic intercalation compounds (of graphite and clay, for example) are known^{6,7}. These observations suggest that small molecules can express significant molecular information when assembled into crystalline structures.

Although cholic acid is a well-known naturally occurring compound, its ability¹ to form inclusion compounds and the channel structure of these was not discovered until 1987. Deoxycholic acid, however, has been known to form inclusion compounds—the choleic acids—since the last century⁸. The compounds differ only in the number of hydroxyl groups in their molecular structures, but their crystal structures differ notably (Fig. 1). The head-to-tail structure of deoxycholic acid results in a rigid sheet consisting of helical hydrogen-bonded networks, as indicated by the constant unit dimensions of the sheet⁹. By contrast, the tail-to-tail structure allows flexibility in the position of the steroidal side chain, bringing about a dynamical structure of the molecular assemblies, which we describe below.

We obtained prismatic pure crystals (without guests) from acetone solution of cholic acid at 30 ± 5 °C. The crystals are orthorhombic, space group $P2_12_12_1$ with unit-cell dimensions of $a = 16.477(4)$, $b = 8.394(3)$, $c = 16.993(3)$ Å. We solved the structure by the direct method and refined it to $R = 0.057$ ($R =$

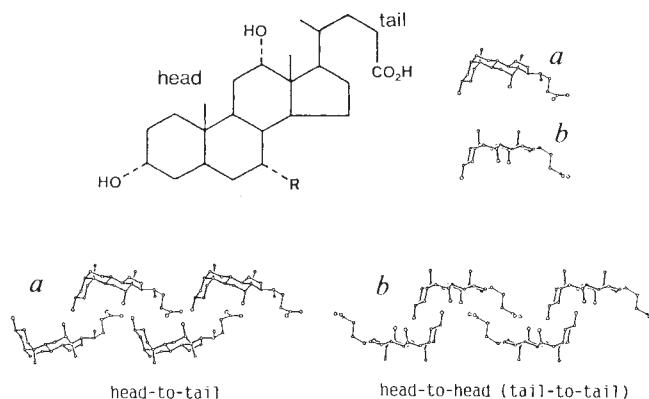


FIG. 1 a, deoxycholic acid ($R=H$) and b, cholic acid ($R=OH$). Two types of their arrangements in crystal structures of the inclusion compounds.

$\Sigma[|F_{\text{obs}}| - |F_{\text{calc}}|]/\Sigma|F_{\text{obs}}|$ where F_{obs} and F_{calc} are the observed and calculated structure factors) using 1,807 observed reflections (K. Miki *et al.*, manuscript in preparation). As shown in Fig. 2, the steroidal molecules are linked to each other by four kinds of hydrogen bond, forming pleated sheets that are stacked so as to leave no cavities between them. The steroidal side chains connect adjacent sheets through the carboxylic groups. The hydrogen bonds are arranged in a spiral manner along the crystallographic b -axis.

Using infrared spectroscopy, we found that the host crystals absorbed acetophenone as guest molecules when soaked in that solvent. The rate depends on the size of the crystals and temperature—in case of crystals with 0.5 mm long, it took about one month to obtain guest-saturated crystals at room temperature. The X-ray diffraction study confirmed that the resulting crystals were identical to those obtained by recrystallization²; that is, the structure changed from orthorhombic (space group $P2_12_12_1$) to monoclinic (space group $P2_1$) indicating a crystal-to-crystal transformation during the absorption of the guest molecules. Subsequent structure determination of this crystal ($R = 0.053$ for 2,074 reflections) confirmed that acetophenone molecules were tightly included in exactly the same way as in the crystal obtained by recrystallization. After releasing the included guest by heating under reduced pressure, the infrared spectrum of the crystals was the same as that of the original crystals.

So, cholic acid crystals can spontaneously absorb guest molecules without any transition to amorphous states; the compound remains crystalline during guest absorption. This is contrary to the generally accepted idea that inclusion crystals are usually formed by recrystallization and that they must be destroyed to release the guest molecules. The single crystal-to-crystal transformation means that molecular assemblies of cholic acid can change while retaining their gross structure—dissociation into monomers is not necessary. This transformation enabled us to study the interconversion between these molecular assemblies, which were in the solid state.

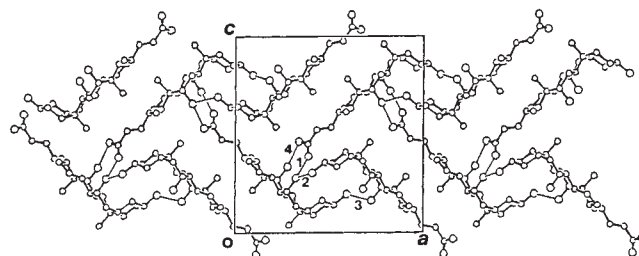


FIG. 2 Crystal structure of cholic acid with no guest molecules, as viewed down along the crystallographic b -axis. Four hydrogen bonds are shown as thin lines numbered 1 to 4.

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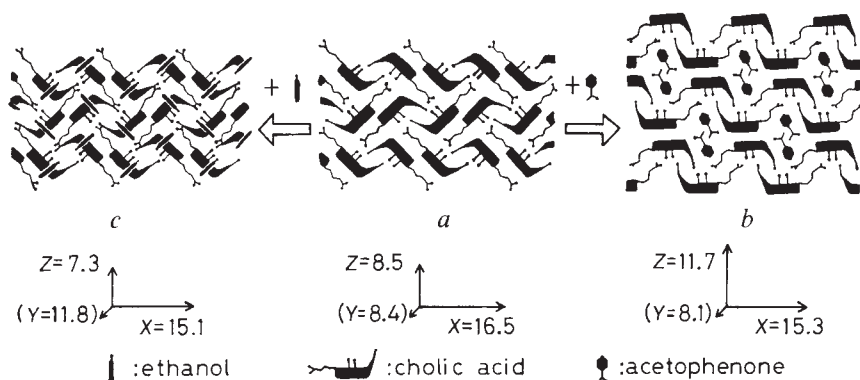


FIG. 3 Schematic representation of intercalation phenomena in cholic acid inclusion crystals as viewed down along the crystallographic *b*-axis. *a*, cholic acid only; *b*, cholic acid/acetophenone; and *c*, cholic acid/ethanol. *x*, *y* and *z* show unit distances (Å) of the axes in the rectangular coordinates.

We therefore interpret the phenomenon as intercalation of the guest molecules between the layers of host molecules. We based this on the comparison of the crystal structures before and after the absorption which reveal the following dynamical behaviour. First, the absorption of acetophenone introduced a drastic change of the crystal structure; the absorbed guests forced the hosts to arrange so that channels appeared. Second, the change in cell dimensions is anisotropic, as shown in Fig. 3; the maximum variation is along the *z*-axis in which, the distance between layers increases from 8.5 Å to 11.7 Å. Third, the carboxylic groups exchanged partners from molecules on the adjacent layer to molecules which used to be their neighbours in the same layer. This results in the hydrogen-bonded network changing from a spiral arrangement to a cyclic one. After the absorption there are only weak van der Waals interactions between the layers. Fourth, the space group changed to P_2 from $P_{21}2_12_1$, indicating that the steroidal molecules have to rotate in one layer but not in the neighbouring layers.

In case of ethanol, we confirmed the transformation by infrared spectroscopy. We believe that the ethanol molecules join the hydrogen-bonded networks and extend the crystallographic *b*-axis from 8.4 Å to 11.8 Å. There are no channels in this structure.

We have not found such a phenomenon in the case of deoxycholic acid. This is due to the fact that the host molecules form rigid and stable sheets of head-to-tail associations, and that the pure host crystal structure remains unknown,—the postulated structure containing no channel is based on theoretical calculation⁹. Therefore, cholic and deoxycholic acid provide an example of the great difference in the molecular assembly structure that can arise from a small difference in molecular structure.

We observed that the assembly of cholic acid can recognize organic guests in size, shape, polarity and chirality much more efficiently than that of deoxycholic acid. For example, the former can include³ *S*-isomers of γ -methyl or γ -ethyl- γ -butyrolactone³. The X-ray crystal-structure analysis gave direct evidence for the chiral recognition (K. Miki *et al.*, manuscript in preparation), indicating that the channels have chiral side pockets enough for accommodation of the chiral guests. In addition, we find that the accommodation space can change in size and shape through conformational changes of the flexible steroidal side chain and sliding of the layers to accommodate guest molecules. We have already ascertained that cholic acid has more than six different kinds of molecular assembly modes. Guest-responsive molecular assemblies such as these may provide a very simple analogue for studying the guest-responsive behaviour of macromolecules such as antibodies.

We believe that this is the first demonstration of intercalation in organic crystals, as compared with those of inorganic compounds such as graphite and clay. Although it is well known that macromolecules in biological systems acquire large amounts of information in the process of molecular evolution, and express their information as dynamical, reversible, environment-dependent, three-dimensional structures suitable for molecular recognition,

the expression of molecular information by small molecules has not hitherto been discussed. The behaviour of the molecular assemblies of cholic acid mentioned above are conceptually similar to those of the macromolecules. It seems that small molecules such as cholic acid can express their molecular information through the behaviour of the assemblies, but not as single molecules. Perhaps such assemblies of small molecules had a role in the process of molecular evolution.

Further systematic research for the intercalation crystals of organic hosts would be useful for the design of new molecular assemblies with guest-responsive and dynamical structures having accurate molecular recognition for organic compounds. □

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1. Miyata, M., Shibakami, M., Goonewardena, W. & Takemoto, K. *Chem. Lett.* 605–608 (1987).
2. Miki, K. *et al.* *J. Am. chem. Soc.* **110**, 6594–6596 (1988).
3. Miyata, M., Shibakami, M. & Takemoto, K. *JCS chem. Commun.* 655–657 (1988).
4. Johnson, P. L. & Schaefer, J. P. *Acta crystallogr.* **B28**, 3083–3089 (1972).
5. Lessinger, L. *Crystal Struct. Commun.* **11**, 1787–1792 (1982).
6. Whittingham, M. S. & Jacobson, A. J. (eds) *Intercalation Chemistry* (Academic, New York, 1982).
7. Atwood, J. L., Davies, J. E. D. & MacNicol, D. D. (eds) *Inclusion Compounds* (Academic, London, 1984).
8. Herndon, W. C. *J. chem. Educ.* **44**, 724–727 (1967).
9. Giglio, E. in *Inclusion Compounds* (eds Atwood, J. L., Davies, J. E. D. & MacNicol, D. D.) Vol. 2, 207 (Academic, London, 1984).

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Sedimentation of particles from a convecting fluid

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THE sedimentation of particles from a convecting fluid is a process of much interest to engineers, fluid dynamicists, geologists and metallurgists. For example, the process plays a fundamental role in controlling the settling behaviour of phenocrysts in magma chambers, crystals and impurities in metallic castings and carbon microparticles in combustion chambers. Previous studies of particle settling in convecting systems^{1–5} have assumed that the concentration of particles is so small that their presence does not modify the convective motion. Here we present experimental results which show that convective motion due to heating from below can be affected by the presence of particles and is controlled by the bulk density of a particulate suspension, which is a function of

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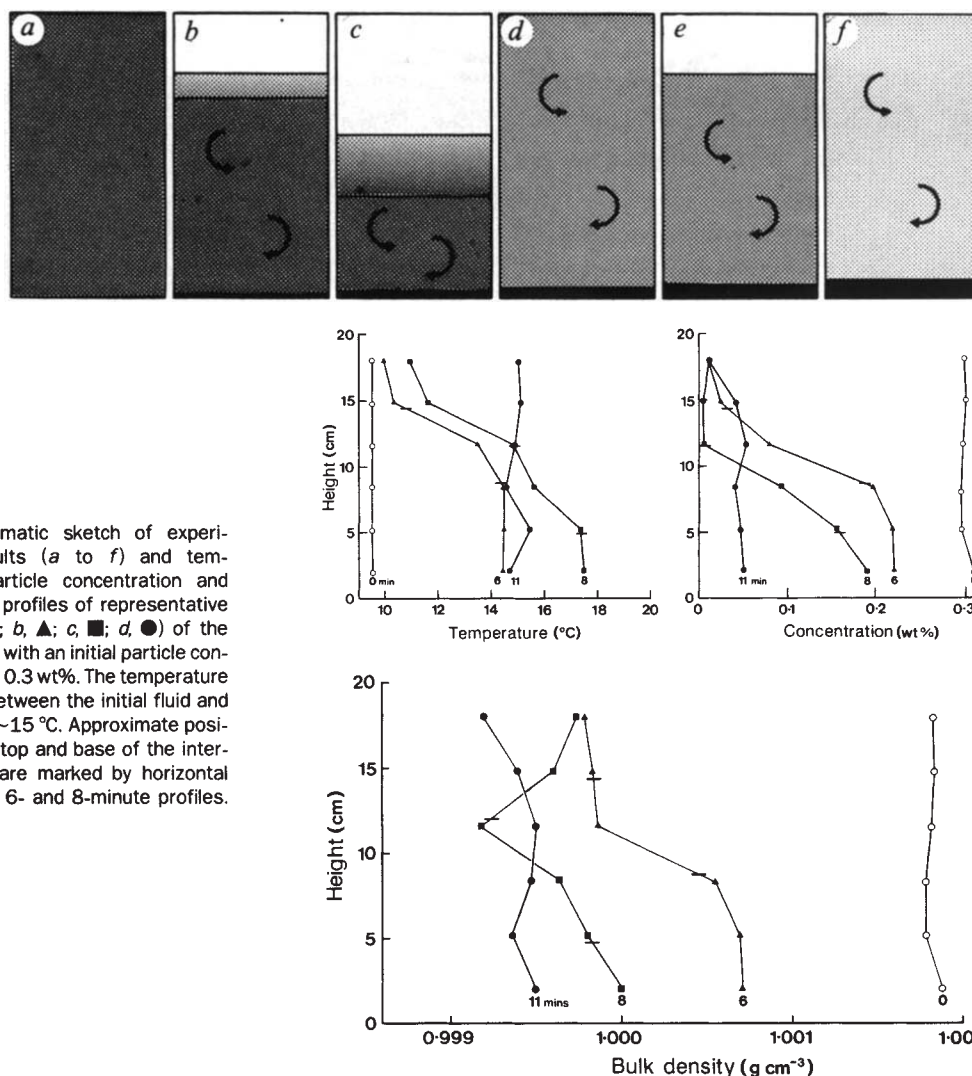


FIG. 1 Schematic sketch of experimental results (a to f) and temperature, particle concentration and bulk density profiles of representative stages a, ○; b, ▲; c, ■; d, ● of the experiments with an initial particle concentration of 0.3 wt%. The temperature difference between the initial fluid and the base is $\sim 15^\circ\text{C}$. Approximate positions of the top and base of the interfacial zone are marked by horizontal bars for the 6- and 8-minute profiles.

both temperature and particle concentration. Above a certain concentration, the particle distribution initially stabilizes the bulk density gradient during sedimentation, and convection is confined to a sedimenting layer beneath a clear layer. Eventually, however, the destabilizing thermal gradient exceeds the stabilizing effect of the particles, and a sudden overturn of the whole system results. Our experimental results agree with a simple physical model.

We carried out experiments in a tank ($20 \times 20 \times 20$ cm deep) heated from below. The tank was filled with a mixture of water and well sorted silicon carbide powder ($16 \mu\text{m}$ in median diameter with a standard deviation of $\sim 3 \mu\text{m}$, particle density $\rho_p = 3.217 \text{ g cm}^{-3}$). The Stokes settling velocity for this range of particle size is $0.02\text{--}0.05 \text{ cm s}^{-1}$, giving a Reynolds number of $\sim 10^{-3}$. The initial temperature difference between the fluid and the base was typically $5\text{--}15^\circ\text{C}$, which leads to Rayleigh numbers of up to 10^9 . The particles were initially distributed uniformly throughout the tank and were then left to settle. Temperatures and particle concentrations were monitored at several heights by thermistors and an optical transmission device⁶ respectively. At least two distinct regimes of settling behaviour were observed for different initial particle concentrations.

At very low initial concentrations (<0.01 wt%), particles were distributed nearly uniformly throughout the tank; deposition occurred at the base and the particle concentration decreased exponentially with time⁵. In our experiments with higher (but still fairly small) initial particle concentrations (~ 0.3 wt%), turbulent convection throughout the whole tank was suppressed

and confined to a suspension layer beneath a sharply defined descending interface.

Schematic sketches and profiles of temperature, particle concentration and bulk density for representative stages of the experiment are shown in Fig. 1. Settling of particles created a clear layer separated from the underlying suspension layer by an interfacial zone (Fig. 1b). The interfacial zone descended at a rate approximately equal to the Stokes settling velocity, and increased in thickness with time mainly as a result of the small variation in particle size and hence in settling velocity (Fig. 1b, c). In the lower suspension layer, temperature and particle concentrations were nearly uniform because of convective mixing; the temperature increased and the particle concentration decreased with time. In the interfacial zone, the temperature and particle concentration decreased with increasing height. This zone was stagnant, because the stabilizing particle concentration gradient was sufficient to overcome the opposing thermal gradient and thus to produce a stable bulk density stratification. The temperature of the upper clear layer, which was insulated from the lower layer by the stagnant interfacial zone, increased only slightly during this stage.

The bulk density of the lower suspension layer decreased with time, as its temperature increased and its particle concentration decreased (Fig. 1). Furthermore, as the interfacial zone descended and became wider, the density gradient in this zone decreased. Eventually it became unstable, and the whole system overturned (about 10 minutes after the start of the experiment),

re-establishing uniform temperature and concentration profiles throughout the tank (Fig. 1d). The homogenized particle concentration after overturn was much lower (~ 0.05 wt%) than the initial concentration because of sedimentation at the base. This cycle was repeated several times (Fig. 1e,f), with the homogenized concentration decreasing after each overturn and with the time interval between each overturn decreasing. Eventually, the concentration became so low that particles settled with uniform concentrations and temperatures throughout the tank, in the same way as in the low-particle-concentration regime.

We have developed a simple physical model of this phenomenon based on the experimental observations. We consider the particles to be of uniform size and to be suspended in a fluid undergoing vigorous convection by being heated from the base at a constant temperature, T_b . The suspension layer decreases in depth with time, leaving a clear layer above. When the volume fraction of particles is small, the descent rate can be approximated by the Stokes settling velocity for a single particle, v_s . We assume that there is a sharp interface between the two layers, which thermally insulates the upper clear layer from the lower suspension layer. The temperature of the upper layer is at the initial value, T_0 , and its density is thus at the initial fluid density, ρ_i .

The bulk density of the lower layer, ρ_l , can be expressed as

$$\frac{1}{\rho_l} = \frac{C}{\rho_p} + \frac{1-C}{\rho_i\{1-\alpha(T_l-T_0)\}} \quad (1)$$

where C is the particle concentration expressed as a mass fraction, ρ_p is the density of the particles, α is the coefficient of thermal expansion and T_l is the temperature of the lower layer. The density of the lower layer decreases with time as T_l increases. When it becomes less than the density of the upper layer, ρ_i , overturn occurs. When $C \ll 1$, the critical temperature at the overturn, T_l^* , is approximated from equation (1) as

$$T_l^* = \frac{C(\rho_p - \rho_i)}{\alpha\rho_p} + T_0 \quad (2)$$

Under the above conditions, the heat flux from the base to the lower layer can be expressed as⁷

$$q = \rho_l c_p \gamma \left(\frac{\alpha g \kappa^2}{\nu} \right)^{1/3} (T_b - T_l)^{4/3} \quad (3)$$

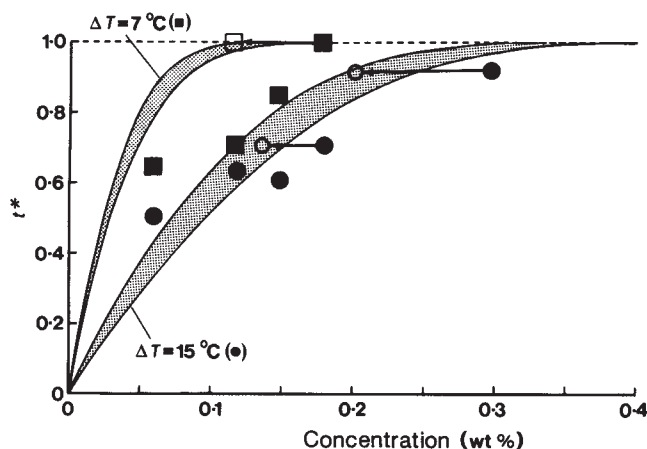


FIG. 2 Theoretical and experimental values of normalized timescale for overturn ($t^* = v_s t / h_0$) plotted against particle concentration for two temperature differences between the initial fluid and the base (●, experiments with $\Delta T = 15^\circ\text{C}$; ■, experiments with $\Delta T = 7^\circ\text{C}$). The shaded regions envelope the theoretically calculated values for a reasonable range of settling velocities, that is, $0.025\text{--}0.035\text{ cm s}^{-1}$ when $\Delta T = 15^\circ\text{C}$ and $0.030\text{--}0.040\text{ cm s}^{-1}$ when $\Delta T = 7^\circ\text{C}$. Arrows from solid to open symbols indicate (where measured) the decrease in concentration of the lower layer from the initial value (solid) to that just before overturn (open).

where ρ_l is the fluid density, c_p is the specific heat, κ is the thermal diffusivity, g is the acceleration due to gravity, ν is the kinematic viscosity and γ is a dimensionless constant, the value of which is determined empirically to be ~ 0.1 at relatively high Rayleigh numbers⁸. The heat conservation equation for the lower layer is

$$q = c_p \rho_l (h_0 - v_s t) \frac{dT_l}{dt} \quad (4)$$

where h_0 is the height of the fluid layer. From equations (3) and (4), the temperature of the lower layer as a function of time can be expressed as

$$T_l(t) = T_b - \left\{ -\frac{\gamma}{3v_s} \left(\frac{\alpha g \kappa^2}{\nu} \right)^{1/3} \ln \left(1 - \frac{v_s t}{h_0} \right) + (T_b - T_0)^{-1/3} \right\}^{-3} \quad (5)$$

The temperature, T_l , increases with time to a critical value, T_l^* , and then overturn occurs.

We define t^* ($= v_s t / h_0$) as the timescale for overturn divided by the timescale for the interface to reach the base, which is a suitably normalized timescale for overturn. From equations (2) and (5), t^* is given by

$$t^* = 1 - \exp \left[-\frac{3v_s}{\gamma} \left(\frac{\nu}{\alpha g \kappa^2} \right)^{1/3} \times \{ (T_b - T_l^*)^{-1/3} - (T_b - T_0)^{-1/3} \} \right] \quad (6)$$

This quantity provides a criterion for the overturn behaviour of the fluid: if $t^* \ll 1$, overturns occur continuously, whereas if $t^* > 1$, overturn does not occur before all the particles have reached the floor.

In Fig. 2 we plot calculated and experimentally determined values of t^* against particle concentration for two specific initial temperature differences between the fluid and the base. The model calculations agree fairly well with the experiments. In our idealized model, particle concentration in the lower layer is constant, because particles will settle out with the same velocity as the interface descends. In reality, however, this concentration decreases with time, mainly because of variation in particle size. The interface velocity lies somewhere between the settling velocities of the smallest and largest particles, so that the larger particles are progressively removed from the sedimenting layer⁹. The agreement between theory and experiment is improved if the concentration just before overturn is used rather than the initial concentration. The timescale increases with increasing particle concentration and with decreasing temperature difference between the base and fluid. When the particle concentration is very small, $T_l^* \approx T_0$ (see equation (2)), and hence t^* is small (see equation (6)). As C increases, $T_l^* \approx T_b$, and hence t^* approaches 1. Because T_l^* cannot be greater than T_b , overturn does not occur if

$$C > \frac{\alpha(T_b - T_0)\rho_p}{\rho_p - \rho_i} \equiv \frac{\alpha\Delta T}{r_p} \quad (7)$$

where r_p is $(\rho_p - \rho_i)/\rho_p$. This implies that $t^* < 1$ only when the particle concentration is so low that the increase in bulk density due to particle suspension ($C(\rho_p - \rho_i)/\rho_p \equiv Cr_p$) is compensated by the decrease in fluid density due to increasing temperature at the bottom ($\alpha(T_b - T_0) \equiv \alpha\Delta T$).

These experimental results indicate an important feature of sedimentation that has not been hitherto recognized. The settling behaviour of particles has previously been accounted for only in terms of the ratio of the particle settling velocity to the vertical component of convective velocity. If the convective velocity exceeds the settling velocity, particles behave like passive tracers of the fluid motion³⁻⁵. Our results suggest, however, that an

additional factor needs to be taken into account, namely the ratio of the decrease in fluid density due to increasing temperature ($\alpha\Delta T$) to the increase in bulk density due to particle suspension (Cr_p).

Although the experiments were carried out in a simplified system, the basic principle expressed by equation (7) is believed to be applicable to natural situations. The main difference is that in our experiments convection is driven by heating from below whereas in natural systems (such as magma chambers) it can be driven both thermally and compositionally at the roof, floor and side walls. Preliminary experiments involving cooling from above suggest that compositional and thermal gradients are established and can lead to overturn and rehomogenization of the particle-laden fluid in a manner similar to that of heating from below. There are some important quantitative differences, however, between the two cases, such as the strength of convection in the upper layer and the thermal evolution of both layers. In the geological context our results suggest that the presence of relatively small amounts of phenocrysts can have a significant influence on the convective motion in a magma chamber, and can provide a mechanism for periodic overturn and rehomogenization of thermal and compositional gradients in an evolving magma body. Such phenomena may also have important consequences for the settling of crystals or impurities in metallurgical casting processes. □

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1. Bartlett, R. W. *Am. J. Sci.* **267**, 1967–1982 (1969).
2. Huppert, H. E. & Sparks, R. S. J. *Contr. Miner. Petrol.* **75**, 279–289 (1980).
3. Marsh, B. D. & Maxey, M. R. *J. Volcanol. geotherm. Res.* **24**, 95–150 (1985).
4. Weinstein, S. A., Yuen, D. A. & Olson, P. L. *Earth planet. Sci. Lett.* **87**, 237–248 (1988).
5. Martin, D. & Nokes, R. *Nature* **332**, 534–536 (1988).
6. Davis, R. H. & Hassen, M. A. *J. Fluid Mech.* **196**, 107–134 (1988).
7. Turner, J. S. *Buoyancy Effects in Fluids* (Cambridge University Press, 1973).
8. Denton, R. A. & Woods, I. R. *Int. J. Heat Mass Transfer* **22**, 1330–1346 (1979).
9. Davis, R. H. & Birdsell, K. H. *A.I.Ch.E. J.* **34**, 123–129 (1988).

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Evidence from rare gases for magma-chamber degassing of highly evolved mid-ocean-ridge basalt

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HIGHLY evolved lavas have been recovered from moderate- to fast-spreading centres along the mid-ocean ridge system, most commonly near propagating rifts and overlapping spreading centres^{1–7}. Their origin and occurrence are generally explained by extensive modification of a primary basalt magma by fractional crystallization at shallow levels in the oceanic crust (model 1)^{1–4,6–9}. Other crustal processes have also been invoked, including periodic magma mixing of a highly fractionated liquid with multiple primary liquids (model 2)^{10,11}, and seawater interaction with the mantle-derived magma (model 3)^{12–14}. Here we report the first ⁴He and argon isotope data for several highly evolved mid-ocean-ridge basalts (MORBs) and andesites. The samples were recovered with the submersible *Alvin* from a very limited section (<8 km²) of the axial valley of the eastern Galapagos Rift, between 85°52' and 85°50' W. Although they range in composition from moderately evolved normal (N) MORB to ferrobasalts, Fe–Ti basalts and high-silica andesites, geochemical studies indicate that the suite

is cogenetic, facilitating interpretation of the data¹⁵. We find distinct differences in the rare-gas data between the evolved and N-MORBs, indicating that the evolved magmas have undergone pre-eruption degassing in a process not common to the source magmas for normal MORBs. Our data fit well with model 1, but provide no evidence for models 2 and 3.

It is well known that the rare gases in N-MORB glasses are dominated by a component derived from the mantle source, because their isotopic composition is distinctly different from crustal materials. Most notably, they are characterized by high ³He/⁴He and ⁴⁰Ar/³⁶Ar relative to the atmosphere. Also, N-MORBs typically have large amounts of ⁴He and ⁴⁰Ar which cannot be accounted for by decay of U, Th and K after eruption, and ⁴He/⁴⁰Ar ratios greater than the production ratio of 1–3. (Subaerial rocks have ⁴He/⁴⁰Ar ratios lower than the production ratio because of diffusive loss.) Typical MORBs have ⁴He contents of 10^{–5}–10^{–6} cm³ STP g^{–1} and ⁴⁰Ar_r contents of ~10^{–6} cm³ STP g^{–1} (where the subscript r refers to the radiogenic component), with ⁴He/⁴⁰Ar_r ratios of 5–25 (ref. 16 and references therein). The N-MORB samples reported here fit this pattern (Table 1), which shows that the Galapagos MORB source is typical of those previously sampled (ref. 16 and references therein). The evolved samples, however, have ⁴He contents of only 10^{–8} cm³ STP g^{–1}, ⁴⁰Ar_r contents of ~10^{–7} cm³ STP g^{–1} and ⁴He/⁴⁰Ar_r ratios of <0.1. The low absolute values measured in the evolved samples indicate loss of mantle gases (relative to N-MORB). The low ⁴He/⁴⁰Ar_r ratios indicate that diffusive loss is the most likely process.

Loss of the mantle gases in the evolved samples is also demonstrated in Fig. 1, in which ⁴⁰Ar/³⁶Ar is plotted against 1/³⁶Ar. If the measured gases consist of a constant amount of trapped mantle ⁴⁰Ar mixed with varying amounts of an atmospheric component, the data should be described by a straight line with an intercept of 295 (ref. 16). Previously measured MORB glasses from a variety of locations are well fit by straight lines¹⁶, as are the three N-MORB measured here ($r^2 = 0.998$, where r is the correlation coefficient), indicating that the total argon components do consist of a single mantle component mixed with varying amounts an atmospheric-like component. The data from the evolved samples are not as well fit by a straight line ($r^2 = 0.83$) and the slope is significantly lower. This indicates a loss of the mantle component variably from sample to sample combined with a variable atmospheric component, and lower trapped ⁴⁰Ar, respectively. Total amounts of the atmospheric component, as measured by the total ³⁶Ar contents, are well within the range of all MORB data¹⁶ and do not vary

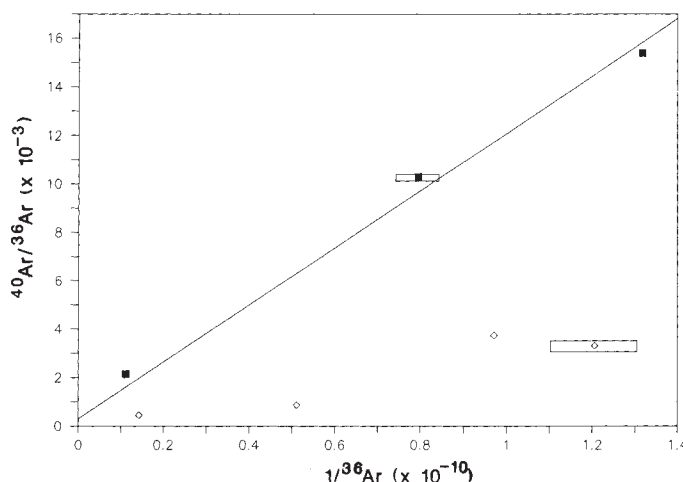


FIG. 1 ⁴⁰Ar/³⁶Ar plotted against 1/³⁶Ar. The straight line is the least-squares fit to the MORB points (■), constrained to pass through the atmospheric ratio of 295. The evolved basalts (◇) lie below the line. Circumscribed rectangles indicate limits of error on typical data points.

TABLE 1 Helium and argon data

Sample	^4He	^{40}Ar	$^{40}\text{Ar}_r$ ($10^{-8}\text{ cm}^3\text{ STP g}^{-1}$)	^{36}Ar	$^{40}\text{Ar}/^{36}\text{Ar}$	$^4\text{He}/^{40}\text{Ar}_r$
N-MORB						
1646-1A	1,054	117	115	0.0076	15,395	9.2
1652-11	790	193	166	0.09	2,145	4.8
1663-6e	1,015	130	126	0.013	10,296	8.1
Andesites						
1652-1GR	1.5	32	12	0.07	459	0.12
1654-3M	2	27	25	0.0083	3,310	0.08
1652-4	1	17	11	0.02	870	0.09
Fe-Ti						
1654-6	0.85	29	26	0.01	3,730	0.03

significantly between the two groups measured here. (The ^{36}Ar is dominated by the atmospheric component, with only a negligible mantle contribution¹⁶.)

Very little of the radiogenic gases in either the N-MORBs or the evolved lavas could have been formed *in situ*. Uranium and thorium have been measured in some of these samples¹⁷. The uranium content of sample 1654 is 0.54 p.p.m. and its Th/U ratio is ~ 3.1 . We calculate that the measured content of ^4He would be generated in $\sim 50,000$ years but, on the basis of maximum sediment cover on sample positions within the axial valley and estimated spreading rates, the samples are less than 15,000 years old. The discrepancy for the other samples, which have more ^4He , is even larger. It would take ~ 100 Myr to generate the measured ^{40}Ar contents *in situ*. We conclude that most (at least) of the radiogenic helium and virtually all the radiogenic argon were trapped from the source region.

Elevated incompatible-element abundances and strontium isotope ratios in the most evolved lavas from this portion of the ridge have been difficult to reconcile with simple closed-system fractionation of an N-MORB parent¹. In view of the proximity of this section of the ridge to the Galapagos hotspot, one might suggest that these samples have been enriched by open-system fractionation and mixing with different mantle components. But Fig. 2, which shows the $^4\text{He}/^{36}\text{Ar}$ ratio plotted against $^{40}\text{Ar}/^{36}\text{Ar}$ for these samples, provides no evidence for this. If the evolved MORBs were formed by mixing of an N-MORB mantle source with an enriched mantle source, and if the enriched source has argon characteristics represented by ocean island basalt (OIB) data (that is, $^{40}\text{Ar}/^{36}\text{Ar} \approx 300$), as claimed by Allegre *et al.*¹⁸, we would expect the data to lie between these two groups in Fig. 2, that is, to lie along the line shown connecting these data.

Clearly they do not. Likewise, if the evolved lavas formed solely by MORB fractional crystallization and assimilation of sea water¹⁴ or seawater-altered crustal materials¹², we would expect them to lie between the seawater-altered basalt cluster and the N-MORB point. Because the altered basalt data are identical, within error, with the OIB data, the conclusion is identical: the N-MORB data do not lie along the connecting line. Thus we find no evidence in these data to support models 2 or 3. We conclude that, although these types of processes may contribute to the genesis of the evolved basalts, they cannot be of primary importance.

The data from the evolved samples indicate the loss of source-generated radiogenic gases (degassing) and the incorporation of an atmospheric-like component. If the degassing was accomplished by venting of a gas phase, all the rare gases would have been lost in roughly equal proportions because all of their solubilities are much greater in the gas phase than in the liquid. Instead, the low $^4\text{He}/^{40}\text{Ar}_r$ ratios indicate a diffusive mechanism. (The similarity of the ^{36}Ar contents is not evidence against this, because the ^{36}Ar measured in the samples does not represent a gas trapped from the source region.) Furthermore, there is no indication of a general loss of volatiles and indeed most volatiles (such as H_2O , Cl and F) in these samples are enriched to the same or greater extent as nonvolatile incompatible elements (that is, Zr, Rb and rare-earth elements). We therefore suggest a diffusive degassing mechanism, with a loss rate of $\text{He} > \text{Ar} >$ less volatile elements.

It is interesting to speculate on when this degassing loss occurred. The helium may have been lost after eruption, but the low absolute concentrations of $^{40}\text{Ar}_r$ are difficult to reconcile with such a mechanism because glasses of various chemistries all have low argon diffusivity rates, particularly at the low ambient bottom-seawater temperatures¹⁶. Such a postulated large argon diffusivity would also have resulted in seawater argon exchange, and would be expected to have led to higher ^{36}Ar concentrations than those in the argon-tight MORBs; an effect which is not seen. Finally, the data for samples that had undergone such a process would plot near the seawater-altered basalts in Fig. 2, or on a mixing line between them and the N-MORBs, and they do not. We therefore favour the hypothesis that degassing preceded eruption.

Previous work on samples from this area indicates that they are cogenetic and that most of the observed petrological and geochemical variability can be explained by extensive ($>90\%$) low-pressure fractional crystallization of an N-MORB magma^{4,6,8,15,19}. Perfit *et al.*¹ favoured the hypothesis that

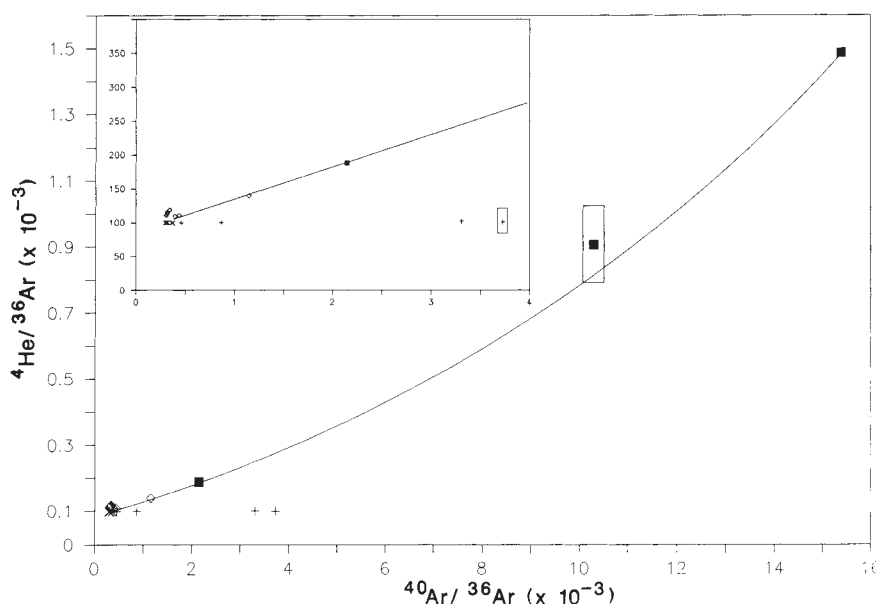


FIG. 2 $^4\text{He}/^{36}\text{Ar}$ plotted against $^{40}\text{Ar}/^{36}\text{Ar}$ for MORB (■), evolved basalts (+), oceanic island basalts (◇), and altered non-glassy MORB (×). Data for OIB from refs 18, 20; data for altered MORB from ref. 16. Insert shows in detail the region below $^{40}\text{Ar}/^{36}\text{Ar} = 4,000$.

differentiated magmas (Fe-Ti basalts and andesites) were separated from parental MORBs after moderate amounts of crystallization and continued to evolve with little mixing with primitive melts. Volatile and isotope data indicate slight (<10%) amounts of assimilation of seawater-altered crust during crystallization of the most evolved liquids^{12,13}. (The fact that no similar indication is seen in the rare-gas data indicates subsequent degassing; see below.) The isolation of these magmas from steady-state magma chambers can explain, in part, the difference between rare-gas abundances in MORBs and highly evolved rock types. We suggest that degassing occurs in all MORBs before eruption, but that it is accentuated in the small, ephemeral magma chambers.

The similar atmospheric argon component (³⁶Ar) in the normal and evolved basalts studied here indicates that it was a late addition rather than being mixed into the mantle source region before degassing. If the latter had occurred, the evolved basalts would show a depletion of ³⁶Ar similar to that observed for the radiogenic rare-gas isotopes. Instead, the equal amounts of ³⁶Ar found in the two groups of samples indicate a similar mechanism for incorporation of the atmospheric component, possibly by stoping and assimilation. The evolved basalts are distinguished from the MORBs by their pre-eruption degassing history rather than by the manner in which they incorporated their atmospheric components. We therefore suggest that the loss of the mantle component exhibited by the evolved samples occurred during a part of their history not shared with the MORBs, perhaps during storage and solidification in a near-

crustal environment, whereas incorporation of the atmospheric-like component occurred during a process common to them both, that is, during intrusion into the oceanic crust. We find no evidence in these data that the interaction of sea water with primary magmas formed the evolved basalts—that is, we see no such interaction beyond that involved in N-MORB generation. We emphasize, however, that the rare-gas data cannot rule out a minor contribution from such processes. □

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1. Perfit, M. R., Fornari, D. J., Malahoff, A. & Embley, R. W. *J. geophys. Res.* **88**, 10551–10572 (1983).
2. Natland, J. H. *Init. Rep. DSDP Leg 54* 833–850 (1980).
3. Clague, D. A., Frey, F. A., Thompson, G. & Rindge, S. *J. geophys. Res.* **86**, 9469–9482 (1981).
4. Christie, D. M. & Sinton, J. M. *Earth planet. Sci. Lett.* **56**, 321–335 (1981).
5. Schilling, J. F., Anderson, R. N. & Vogt, P. *Nature* **261**, 108–113 (1976).
6. Byerly, G. *J. geophys. Res.* **85**, 3797–3810 (1980).
7. Bender, J., Langmuir, C., Natland, J. & Batiza, R. *Eos* **67**, 1254 (1982).
8. Perfit, M. R. & Fornari, D. J. *J. geophys. Res.* **88**, 10530–10550 (1983).
9. Natland, J., Langmuir, C., Bender, J., Batiza, R. & Hopson, C. *Eos* **67**, 1254 (1986).
10. O'Hara, M. J. *Nature* **266**, 503–507 (1977).
11. O'Hara, M. J. & Mathews, R. E. *J. geol. Soc. Lond.* **138**, 237–277 (1981).
12. Cornell, W. C., Taylor, B. E. & Perfit, M. R. *New Mex. Bureau Mines Min. Res.* **131**, 61 (1989).
13. Jonasson, I. R., Perfit, M. R. & Gregoire, D. C. *Nature* (submitted).
14. Michael, P. & Schilling, J.-G. (abstr.) *Eos* **69**, 1469 (1988).
15. Embley, R. W. *Can. Mineralogist* **26**, 517–539 (1988).
16. Fisher, D. E. *Geochim. cosmochim. Acta* **50**, 2531–2541 (1986).
17. Williams, R. W. & Gill, J. S. *Geochim. cosmochim. Acta* (in the press).
18. Allegre, C. J., Staudacher, T., Sarda, P. & Kurz, M. *Nature* **303**, 762–766 (1983).
19. Juster, T. C., Grove, T. L. & Perfit, M. R. *J. geophys. Res.* **94**, 9251–9279 (1986).
20. Hart, R., Dymond, J., Hogan, L. & Schilling, J.-G. *Nature* **305**, 403–407 (1983).

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A new mechanism of granite emplacement: intrusion in active extensional shear zones

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ONE of the principal ways in which continental crust grows is by the incorporation, from deeper within the Earth, of large volumes of granitic magma. A 'space problem' exists¹ as to how these magmas are accommodated in the crust. Traditionally two main emplacement mechanisms have been emphasized: 'forceful' intrusion, whereby buoyancy-driven magmas physically push the crust aside, creating granitic diapirs and balloons; and 'passive' emplacement characterized by replacive mechanisms such as cauldron subsidence and stoping. Although more recent work^{2,3} has demonstrated that space for granites may be created within bends and offsets of large transcurrent faults, the simple view of either forceful or passive still fails to account for the intrusive mechanisms of many granites. Here we report the discovery of a new igneous intrusion mechanism. In the superbly exposed Proterozoic continental crust of South Greenland we have observed that rapakivi granite was intruded as large-scale sheets along ductile extensional shear zones that were active during emplacement. In such a process the space problem seems to be simply resolved.

Our data come from the voluminous rapakivi granite suite of southern Greenland (Fig. 1). Reviews of the petrogenesis and significance of these early Proterozoic intrusions with their enigmatic large ovoid and mantled feldspars may be found elsewhere^{4,5}. Although we comment later on the general

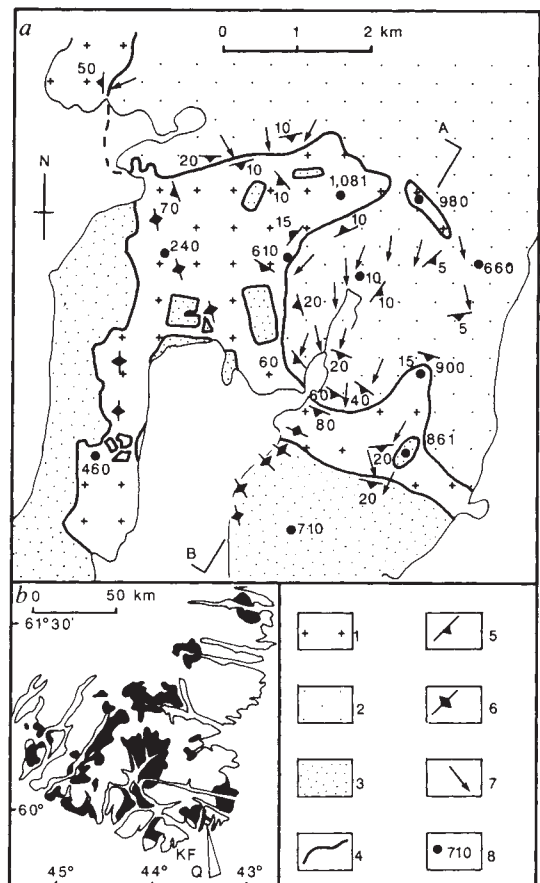


FIG. 1. a, Geological map of western Qernertoq Island. Ornament: 1, Rapakivi granite; 2, migmatites and schists; 3, microcline granite; 4, contact of rapakivi granite with 2 and 3; 5, strike and dip direction of inclined-shear-zone foliation; 6, vertical-shear-zone foliation; 7, direction of overshear in shear zone; 8, spot heights (metres). A-B is section line for Fig. 3. b, Inset map of southernmost Greenland: KF, Kap Farvel; Q, Qernertoq Island; A, Augpilagtoq; Black ornament, rapakivi intrusions¹⁸.

implications of our data for the rapakivi province, our primary purpose here is to treat them simply as members of the granite family the emplacement styles of which may be applicable to general granitic magmatism.

The plutons occur in Greenland as flat- and steep-sided bodies of 1,755–1,740 Myr age (refs 6, 7) which were emplaced into country rocks previously deformed and metamorphosed at ~1,800 Myr (refs 6, 7) during the Ketilidian orogeny. The regional structure before rapakivi granite emplacement was one of flat-lying or gently inclined upper amphibolite to granulite-facies migmatites and schists overlain by a regionally developed and concordant microcline granite sheet ~1 km thick⁸. On southern Qernertoq Island (Fig. 1) 40 km north-east of Kap Farvel, the relationship between one of the rapakivi intrusions and the structure of the country rock can be examined in nearly complete three-dimensional, glaciated exposure with over 1 km

of relief (Fig. 2). At high levels in the structure (to the north) the rapakivi is a flat or gently southwards-inclined sheet, ~400 m thick, which has intruded along the interdigitated boundary between the underlying schists and migmatites and the overlying microcline granite. Farther south, the base of the rapakivi sheet intrudes obliquely downwards across the schist banding and foliation in the footwall, whereas at shore levels the contact is vertical or steeply southwards inclined. In the steep down-cutting segment, although the footwall structures are intrusively and obliquely cross-cut by the granite, they are also deflected downwards and southwards towards concordance with the base of the rapakivi sheet (Fig. 2). The deformation that created this deflection of the footwall banding is normal (or extensional) in character with a sense of shear that was top towards the south. Confirmatory evidence of this type of deformation around the rapakivi sheet can be seen in many

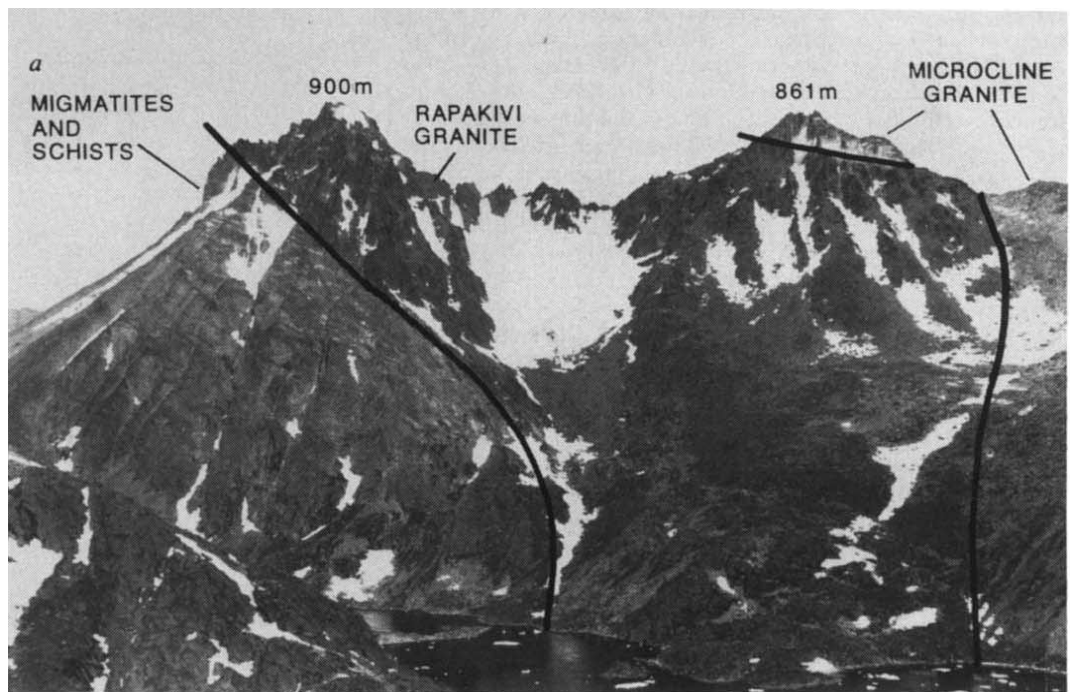


FIG. 2 *a*, View south-east towards peak 900 m (left) and peak 861 m (right) (see Fig. 1*a* for location). This shows dark-coloured rapakivi granite cutting down across banded migmatites in the footwall. Light-coloured microcline granite forms the hanging wall to the rapakivi intrusion. *b*, Close up of peak 900 m showing the oblique intrusion of the base of the rapakivi downwards and across the migmatite banding.



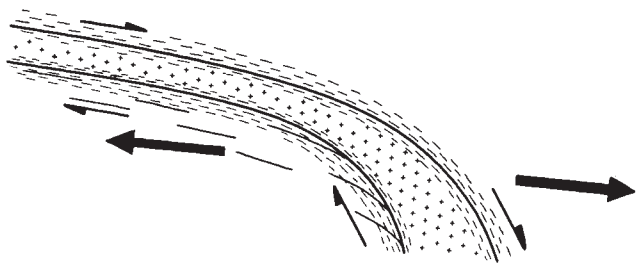


FIG. 3 Partly schematic geological section along A-B in Fig. 1. Solid-geology ornaments as Fig. 1. Solid full arrows show tectonic extension orientated approximately north-south. Solid half arrows show resulting shear sense across shear zone. Short dashes show approximate extent and intensity of shear zone. Long dashes show deflection of pre-existing banding and foliation in migmatites and schists of footwall.

smaller-scale structural features.

Below the upper, gently inclined part of the rapakivi intrusion, pre-existing folds of Ketilidian age are redeformed by a new gently inclined cleavage that is concordant with the base of the sheet. This fabric intensifies upwards over a thickness of ~80 m towards the sheet and it develops a distinctive north-south-trending tectonic stretching lineation. Small-scale sense of shear indicators, such as S-C fabrics (a type of asymmetric crenulating cleavage that can be used to determine slip sense in deformed rocks), discrete extensional shears and asymmetric boudins are well developed in this shear zone and show consistent top-to-the-south relative movement. Mylonites are common and ductilely deformed feldspars in them indicate that the deformation occurred in conditions of the amphibolite facies or above⁹. Farther south, where the footwall banding is deflected downwards towards the base of the cross-cutting rapakivi sheet, similar small and mesoscopic structural features are found and these also show top-to-the-south shear sense. In the microcline granite, which occurs as the hanging wall to the rapakivi intrusion, pre-existing north-south-trending fabrics are progressively redeformed by high-temperature S-C fabrics that are concordant with, and intensify towards, the top of the rapakivi sheet. These again show top-to-the-south movement. Thus large-scale and small-scale structural features are in accord and show that a shear zone of extensional character exists in the country rocks around and concordant with the rapakivi intrusion and that deformation in the shear zone intensifies towards the intrusive sheet.

Critical evidence for the relative timing of the rapakivi granite intrusion and the shear-zone deformation comes from the thermal metamorphic aureole of the granite sheet. We have determined that this aureole, which contains the assemblage garnet, cordierite, biotite, potassium-feldspar $\pm$ orthopyroxene $\pm$ hercynite and for which our preliminary geothermometry and geobarometry indicate conditions of 700 °C and 2 kbar, is coincident in space with the highly deformed country rocks of the extensional shear zone. The high-temperature deformation of the microcline granite feldspars, noted above, and mineral growth features (such as the fact that garnets in the aureole may both overprint S-C fabrics and be deformed by them) indicate that the deformation and the intrusion occurred at the same time. The rapakivi body is itself deformed by the shear zone. This may take the form of localized intense solid-state¹⁰ or crystal plastic strain^{11,12} fabrics and top-to-the-south S-C cleavages at many of the pluton contacts, which indicate that some of the deformation occurred when the rapakivi had crystallized but was still hot. Alternatively rapakivi fabrics may be of pre-full-crystallization type^{11,12} indicating that shear-zone deformation initially occurred while the magma was largely uncrystallized. We found further evidence of general synchronization where a 10-m-thick late-stage rapakivi sheet had intruded along the contact between the main rapakivi body and the country rocks. This sheet contains xenoliths of strongly deformed main rapakivi but the sheet itself has been weakly

deformed by continuing shear-zone movements. In conclusion these data, which are typical of syn-kinematically emplaced granitoids^{2,13} imply that the intrusion, aureole heating and shear-zone deformation were coeval. Combined with the previously described structural geometry and shear-sense data they indicate that the Qernertoq rapakivi body intruded along the median plane of an active ductile extensional shear zone (Fig. 3).

The geometry of the ductile shear zone in Greenland is strikingly analogous to the brittle fault systems of higher crustal levels found in sedimentary basins, rifts and passive margins elsewhere in the world^{14,15}. For example, a 'ramp' and 'flat' are clearly developed and the flat, on both a local and regional scale, lies along the pre-existing boundary of the migmatites and the microcline granite suggesting that this surface acted as a regional detachment horizon. Further, our mapping shows that in terms of the local setting, the steep ramp of the structure is part of a larger-scale frontal ramp/(sinistral) transfer zone/frontal ramp system (Fig. 4) geometrically and kinematically similar to fault-transfer systems in sedimentary basins^{16,17}.

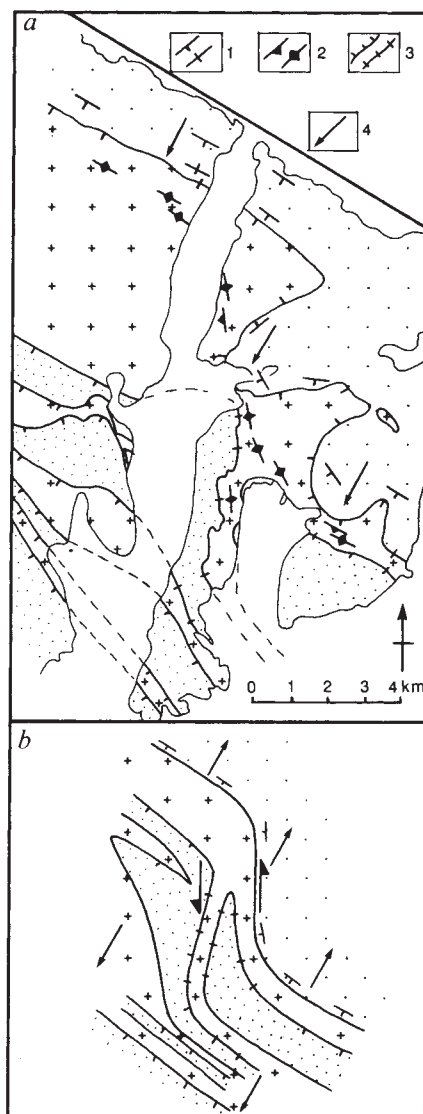


FIG. 4 a, Geology of Qernertoq Island and adjacent mainland. Solid-geology ornaments same as Fig. 1. Key: 1, strike and dip of inclined and vertical pre-existing banding in migmatite/schist footwall to rapakivi intrusion; 2, strike and dip of steeply inclined and vertical-shear-zone foliation in the ramps and transfer zone of the rapakivi intrusion; 3, strike and dip of inclined and vertical rapakivi contacts; 4, direction of overshear. b, Schematic representation of a. Full arrows, direction of tectonic extension; half arrows, implied sinistral sense of shear in transfer zone.

With the synchronous involvement of magma the larger-scale structure is also analogous to a ridge-transform-ridge system of an ocean setting.

We consider that the space problem for granite emplacement may be resolved in the Greenland situation. In sedimentary basins, extension using ramp and flat geometries creates space, primarily in the ramp area, and this is usually and immediately transferred away from the fault to produce hanging-wall collapse, roll-over structures and hanging-wall basins¹⁶. In Greenland the space need not have been transferred, but was probably occupied at the place and time of its origin by the intruding magma. Although magma buoyancy forces may have created some more space by elevating the roof of the intrusion, space created during horizontal extension across a steeply inclined ramp linked to a gently inclined flat seems to account for the volume of the intruding magma (Fig. 3).

The general relationship of synchronous magmatic intrusion into extensional shear zones may have wider applications. Previous workers¹⁸ have proposed that the rapakivi bodies in Greenland have 'mushroom' shapes with flat tops overlying a vertical central stem. Although we have not completed our investigations in the entire Greenland rapakivi province, we have found no clear evidence of mushroom geometries in the large and adjacent Aupilaqtoq area (Fig. 1) and the intrusions there seem to have

ramp/flat shapes with bounding transfer zones associated with obvious extensional deformation. This implies that the period of rapakivi intrusion in Greenland was one of regional extension of the early Proterozoic continental crust similar to the scenario developed by previous workers⁶. □

Received 4 August; accepted 6 December 1989.

1. Read, H. H. *Mem. geol. Soc. Am.* **28** (1948).
2. Hutton, D. H. W. *J. geol. Soc. Lond.* **139**, 615–631 (1982).
3. Castro, A. J. *J. struct. Geol.* **8**, 633–645 (1986).
4. Elliston, J. N. *Earth Sci. Rev.* **2**, 1–92 (1985).
5. Bridgwater, D. & Windley, B. F. *Spec. Publ. geol. Soc. S. Afr.* **3**, 307–316 (1973).
6. Allart, J. H. in *The Geology of Greenland* (eds Escher, A. & Watt, W. S.) 120–151 (Grøn. Geol. Unders., city, 1976).
7. Gulson, B. L. & Krogh, T. E. *Geochim. cosmochim. Acta* **39**, 65–82 (1975).
8. Sutton, J. & Watterson, J. *Rapp. Grønlands, Geol. Unders.* **15**, 58–60 (1968).
9. Simpson, C. J. *struct. Geol.* **7**, 503–511 (1985).
10. Blumenfeld, P. & Bouchez, J.-L. *J. struct. Geol.* **10**, 361–372 (1988).
11. Hutton, D. H. W. *Bull. geol. Soc. Am.* **100**, 1392–1399 (1988).
12. Hutton, D. H. W. *Trans. R. Soc. Edinb.* **79**, 245–255 (1988).
13. Pitcher, W. S. & Berger, A. R. *The Geology of Donegal: A Study of Granite Emplacement and Unroofing* (Wiley, London, 1972).
14. Wernicke, B. & Burchfield, B. C. *J. struct. Geol.* **4**, 105–115 (1982).
15. Gibbs, A. D. *J. geol. Lond.* **141**, 609–620 (1984).
16. Gibbs, A. D. *Spec. Publ. geol. Soc. Lond.* **28**, 19–33 (1987).
17. Hossack, J. R. *J. geol. Soc. Lond.* **141**, 629–637 (1984).
18. Bridgwater, D., Sutton, J. & Watterson, J. S. *Tectonophysics* **21**, 57–77 (1974).

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Predator–prey dynamics in environments rich and poor in nutrients

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STABLE predator–prey models with a tension between the stabilizing effect of prey density-dependence and the destabilizing effect of predators generally become unstable when the nutrient environment of the prey is enriched^{1–3}. This 'paradox of enrichment' occurs in a wide range of models incorporating realistic features^{4–6}. Enriched models quickly produce cycles with enormous amplitude and increased periods^{2,3,6–8}. In real systems, however, there have been few tests for 'paradox' behaviour, and the few results are equivocal^{9–12}. We have now tested for paradox behaviour in populations of the freshwater zooplankton *Daphnia* and their algal prey. In lakes and ponds these populations show both stable and cyclic dynamics^{13,14} caused by the interaction between the populations. The cycles are not driven by external forces^{14,15}, but do not seem to be of the paradox-of-enrichment type: their amplitude is small, their period (equal to a *Daphnia* generation) is short, and there is good evidence that they are caused by the developmental delay of *Daphnia*^{14,15}. Stage-structured models of the *Daphnia*–algae interaction indicate that such single-generation 'time-lag' cycles occur in the progression of instability from stable equilibrium to paradox cycles^{6,7}, and that this progression may be driven by increasing nutrient levels⁷. We show here, however, that enrichment does not lead to paradox cycles, either in field or experimental *Daphnia*–algae systems. We suggest that concomitant changes in the predator–prey interaction accompanying enrichment could account for the absence of paradox behaviour.

With existing data and a new experiment on *Daphnia*–algae systems, we addressed two questions. First, do paradox cycles, characterized by a marked increase in amplitude and period, appear in nutrient-rich systems? Second, if not, is there an

increase in the prevalence or amplitude of the time-lag cycles with enrichment, reflecting increased instability?

Analysis of field data shows that no paradox cycles appear (Fig. 1). Also, the correspondence between cycle periods and the generation time of *Daphnia* is consistent with our previous interpretation that they are single-generation time-lag cycles¹⁴.

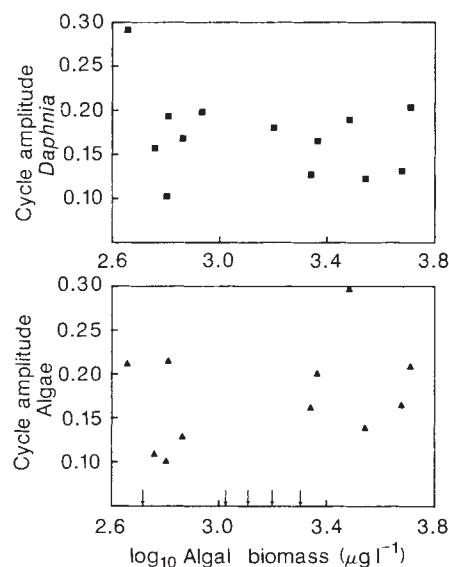


FIG. 1 Cycle amplitudes (log) observed for *Daphnia* and algal populations from lakes and ponds collated by McCauley and Murdoch¹⁴ (Table 2). Comparable studies are plotted (that is, *Daphnia* and algae estimated as density and biomass, respectively). Cycle periods (reported by McCauley and Murdoch; Table 2) are not positively correlated with changes in algal biomass among systems; in fact, the trend was in the opposite direction (product-moment correlation coefficients were negative for both *Daphnia* and algae, but not significantly different from zero $P > 0.05$). The average total algal biomass is used as an index of level of enrichment, because the positive correlation between total algal biomass and phosphorus levels among lakes is well established^{18,19}. Arrows show the average total algal biomass of those systems that are stable (that is, class 1 dynamics^{13–14}).

TABLE 1 Effects of enrichment

Population		Dependent variable	Treatment (poor, 0; rich, 1)	Mean ($\pm$ s.d.)*	n	Parameter estimate†	F value	P > F
<i>Daphnia</i>	Total	s.d. log	0	0.200 (0.034)	6	-0.029	2.5	0.145
		s.d. log	1	0.171 (0.029)	6			
		amplitude	0	0.173 (0.039)	6	-0.046	4.53	0.066
		amplitude	1	0.127 (0.022)	4			
	Juvenile	s.d. log	0	0.170 (0.05)	6	-0.045	3.08	0.11
		s.d. log	1	0.126 (0.037)	6			
		amplitude	0	0.133 (0.017)	5	-0.023	1.18	0.31
		amplitude	1	0.110 (0.043)	5			
Algae	Edible	s.d. log	0	0.178 (0.021)	6	-0.023	3.6	0.087
		s.d. log	1	0.156 (0.020)	6			
		amplitude	0	0.121 (0.030)	4	0.075	3.14	0.12
		amplitude	1	0.196 (0.080)	5			
<i>Daphnia</i>	Total (mg l^{-1})	biomass	0	0.169 (0.076)	6	0.356	6.28	0.031
		biomass	1	0.526 (0.310)	6			
Algae	Edible ($\mu\text{g l}^{-1}$)	biomass	0	5.73 (1.87)	6	20.81	38.8	0.001
		biomass	1	26.5 (7.39)	6			

* Standard deviation.

Results from analysis of variance for the effects of enrichment on changes in dynamics or equilibria. Two estimates of variability were used for *Daphnia* populations and edible algae. First, the s.d. of logarithmic residuals from long-term seasonal trends. Variables, such as *Daphnia* biomass, were first transformed logarithmically, and seasonal trends between log *Daphnia* biomass and time were determined with linear regression. The s.d. of the residuals from these relationships was then used as a measure of variability. Second, the amplitude of harmonic regressions fitted to the residuals from above. This analysis was done exactly as those previously reported for field populations^{13,14}. The relationship

$$\text{residual}(t) = \text{amplitude} \cdot \cos[(2\pi/\text{period}) \cdot (t - \text{phase shift})]$$

was fitted using nonlinear regression. The significance of the cycle was determined by whether the amplitude of the cycle was significantly different from zero (that is, whether the 95% confidence interval for the amplitude overlapped zero). For tests involving the s.d. of the logs and average biomass, the design was balanced, whereas it was unbalanced for amplitudes because the fit of the harmonic regression was insignificant in a few cases (see column n). The significance of effects in all tests was $P=0.05$.

† Model, dependent variable = TRT.

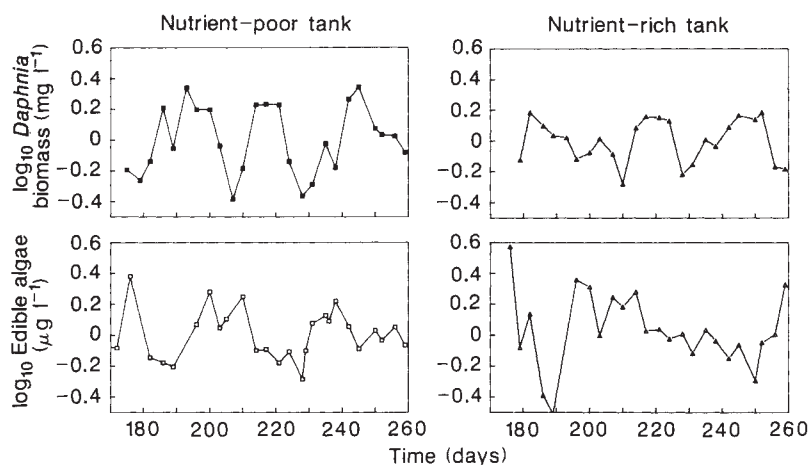
The data also showed that instability, as revealed by the time-lag cycles, does not increase with an increase in nutrient level: stable populations are not restricted to nutrient-poor lakes (Fig. 1), and the cycle amplitude is not related to nutrient level in either *Daphnia* ($P > 0.34$) or algae ($P > 0.38$). In addition, both stable and cyclic populations appear in the same lake in different years (for example, Lakes Washington and Maarsveen), with no reported dramatic change in the nutrient status of the lake¹⁴, and both stable cyclic populations exist in adjacent stock tanks with no nutrient differences¹³.

We raised experimental populations in twelve tanks each of 750 litres, in a greenhouse located beside two ponds on the University of Calgary campus. We filled the tanks in the spring

with pond water that had been filtered (70- μm mesh net) to remove large zooplankton and invertebrate predators (such as *Chaoborus*). The greenhouse environment overcame seasonal variation in temperature, and the tanks contained a diverse plankton assemblage.

Nutrient-poor tanks received oligotrophic pond water (total phosphorus $< 20 \mu\text{g l}^{-1}$) yielding an algal carrying capacity of $300 \mu\text{g l}^{-1}$. Enriched tanks had a carrying capacity more than 10-times higher ($> 3000 \mu\text{g l}^{-1}$). From existing *Daphnia*-algae models, whether simple or complex, and with laboratory-determined parameter values, stable dynamics in the nutrient-poor tanks and large-amplitude paradox cycles in nutrient-rich tanks are predicted.

FIG. 2 Examples of the dynamics of *Daphnia* and edible algal biomass (measured as mg dry weight per l and μg per l chlorophyll *a*, respectively) from nutrient-rich and -poor tanks. Tanks were assigned randomly to one of two treatments: 'poor' tanks simply contained pond water, and 'rich' tanks were enriched with phosphorus, nitrogen and carbon. (Each rich tank received K_2HPO_4 (0.445 g), NH_3NO_3 (4.42 g) and NaHCO_3 (0.645 g).) The level of enrichment was chosen, based on empirical models^{18,19}, to increase the algal carrying capacity by roughly one order of magnitude to $> 3000 \mu\text{g l}^{-1}$. Sampling began 1 week after inoculating tanks with *Daphnia*, and continued twice a week for 4 months. Residual deviations from long-term 'simple' seasonal trends are presented, and the panels have identical scales to facilitate comparisons (time as Julian days). Entire replicate samples of *Daphnia* were counted, and the number of individuals in five size-classes (< 0.8 , 0.8 – 1.0 , 1.1 – 1.6 , 1.7 – 2.0 and 2.0 – 3.0 mm) were recorded. Dry weights for representative individuals of each size-class were determined using length-weight relationships²⁰, and these estimates were used along with their densities to calculate the biomass of *Daphnia* populations in the tanks. Edible algal biomass was estimated from measurements of chlorophyll *a*, the concentration of which in the phytoplankton fraction of



$< 35 \mu\text{m}$ was determined by ethanol extraction followed by spectrophotometric measurements²¹.

After the development of substantial algal biomass in the nutrient-rich systems, we added ~160–200 individuals from a single clone of *Daphnia pulex* to each tank over a 7-day period. Algal biomass was >10-times greater in nutrient-rich tanks than it was in nutrient-poor tanks before we inoculated *Daphnia*. *Daphnia* increased rapidly in abundance, and reduced algal biomass to very low levels, so that after ~2 weeks there was no significant difference in the biomass of edible algae between nutrient-rich and nutrient-poor tanks, although differences appeared later in the experiment. Subsequent changes in the populations reinforce our two conclusions.

First, enrichment does not lead to paradox cycles. *Daphnia* and algal populations displayed similar dynamics in nutrient-rich and -poor tanks (for example, see Fig. 2). In general, *Daphnia* populations showed time-lag cycles in both treatments with amplitudes, periods, and demographic details (data not shown) similar to those observed in field and other tank populations^{13–14}. Second, as for the field data, enrichment did not lead to an increase in the amplitude of time-lag cycles or in the temporal variability of either *Daphnia* or algal biomass; indeed, if anything, the opposite was true (Table 1). The experiment described here thus confirms the cross-habitat comparisons.

These results pose a major dilemma. The paradox of enrichment is a fundamental property of a huge range of predator-prey models, particularly those proposed for the zooplankton-algae interaction. Yet paradox cycles do not occur in this system. What suppresses the cycles? Any explanation must account for the astonishingly uniform dynamics across an enormous range of nutrient levels and their remarkable similarity to those seen in physically simple tank environments. Two hypotheses seem most likely.

First, the paradox assumes that enrichment causes only the environmental carrying capacity for prey (*K*) to increase. Perhaps existing models of the *Daphnia*-algae interaction are appropriate, but parameters other than *K* change in a stabilizing direction; specifically, the attack rate of *Daphnia* decreases because the densities of inedible¹⁶ and larger edible¹⁷ algae increase with enrichment. This can also explain why average prey biomass increases with enrichment (Table 1), whereas the paradox predicts that only predator biomass should increase¹⁵. Also, in a stage-structured model of the *Daphnia*-algae system, reducing the attack parameter suppresses enormous paradox cycles and exposes time-lag cycles of the correct amplitude and period⁶.

Alternatively, some universal stabilizing process occurs that is missing from current models seeking to explain the observed dynamics. This hypothesis, now under investigation, would explain the invariant dynamic patterns seen across the spectrum of environments.

The results presented here have implications for most ecological communities, which contain populations of prey and predators. Are observed cycles in other systems paradox (or more broadly, 'predator-prey') cycles, or time-lag cycles? Are predator-prey cycles associated with richer environments? Predator-prey cycles seem relatively rare in nature: why? Solving the plankton paradox should provide insight into these wide-ranging questions. □

14. McCauley, E. & Murdoch, W. W. *Am. Nat.* **129**, 97–121 (1987).
15. McCauley, E., Murdoch, W. W. & Watson, S. *Am. Nat.* **132**, 383–403 (1988).
16. Watson, S. & Kalff, J. *Can. J. Fish. Aquat. Sci.* **38**, 960–969 (1981).
17. Watson, S. & McCauley, E. *Can. J. Fish. Aquat. Sci.* **45**, 915–920 (1988).
18. Dillon, P. J. & Rigler, F. H. *Limnol. Oceanogr.* **19**, 767–773 (1974).
19. McCauley, E., Downing, J. A. D. & Watson, S. *Can. J. Fish. Aquat. Sci.* **46**, 1171–1175 (1989).
20. Paloheimo, J. E., Crabtree, S. J. & Taylor, W. D. *Can. J. Fish. Aquat. Sci.* **39**, 598–606 (1982).
21. Bergmann, M. & Peters, R. H. *Can. J. Fish. Aquat. Sci.* **37**, 111–114 (1980).

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The *preS2/S* region of integrated hepatitis B virus DNA encodes a transcriptional transactivator

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HEPATITIS B virus (HBV) is regarded as the main aetiological factor in the development of human hepatocellular carcinoma (HCC), one of the most frequent fatal malignancies worldwide^{1,2}. Detection of integrated HBV sequences in the cellular DNA of almost all HCCs studied^{2–17}, and the recent finding that the integrated HBV open reading frame (*orf*) *X* encodes a *trans*-activating activity¹⁸, supports the notion that integrated HBV DNA could contribute to liver carcinogenesis by activation of cellular genes in *trans*^{18–20}. But not all HCCs seem to harbour a functional *orf X*^{2–17}. We report here that 3'-truncated *preS2/S* sequences in integrated HBV DNA of liver cell carcinomas encode a so far unidentified transcriptional *trans*-activation activity. This activity is also produced by an artificially 3'-truncated *preS2/S* gene of the wild-type HBV genome. Besides the simian virus 40 promoter of the reporter plasmid pSV2CAT, the promoter of the human *c-myc* oncogene can also be activated. These results, taken together with the fact that *preS/S* is the only HBV gene found to be integrated in almost every HBV-related HCC analysed so far^{2–17}, indicate that *trans*-activation by integrated *preS2/S* sequences is a possible mechanism for HBV-associated oncogenesis.

The human hepatoma cell line huH-4, positive for the HBV surface antigen (HBsAg)²¹, contains one single 5.6-kilobase (kb) sequence of integrated HBV DNA, which we cloned into the cosmid vector pHC79 (Fig. 1a). Owing to multiple rearrangements, only the *orf X* and the *preS/S* gene are maintained in a functional state. For the detection of possible *trans*-activating activities encoded by the integrated HBV DNA, we constructed the plasmids pHU-a, pHU-b and pHU-c (Fig. 1b) and performed co-transfection experiments with pSV2CAT as a reporter plasmid, encoding the chloramphenicol acetyltransferase gene (*CAT*; ref. 22). Surprisingly, not only pHU-a, which contains the complete integrated HBV DNA and an intact *orf X*, but also the subclones pHU-b and pHU-c, exerted a strong stimulatory effect on expression from the pSV2CAT reporter plasmid (Fig. 2a). Because neither of the two subclones contains a complete *orf X*, this observation indicates the existence of a novel *X*-gene-independent transactivator.

To identify the pSV2CAT-activating region, further subclones of pHU-b were constructed (Figs 1b and 2b). Complete elimination of *orf X* and the recently described *orf 5* (ref. 23) in plasmid pHU-b7 (Fig. 1b) did not impair the stimulation of pSV2CAT (Fig. 2b, lane 6). Also, the additional deletion of 3' cellular

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1. Rosenzweig, M. L. *Science* **171**, 385–387 (1972).
2. May, R. M. *Science* **177**, 900–902 (1972).
3. Gilpin, M. E. *Science* **177**, 902–904 (1972).
4. May, R. M. *Ecology* **54**, 315–325 (1973).
5. Hastings, A. & Wolkind, D. *Theor. Populat. Biol.* **21**, 44–68 (1982).
6. Nisbet, R. M., Gurney, W. S. C., Murdoch, W. W. & McCauley, E. *Biol. J. Linn. Soc. Lond.* **37**, 79–99 (1989).
7. de Roos, A. & Metz, J. A. J. *Ecology* (submitted).
8. Hsu, S. B., Hubbell, S. P. & Waltman, P. *Ecol. Monogr.* **48**, 337–349 (1978).
9. Luckinbill, L. S. *Ecology* **55**, 1142–1147 (1974).
10. McAllister, C. D., Le Brasseur, R. J. & Parsons, T. R. *Science* **175**, 562–564 (1972).
11. Rosenzweig, M. L. *Science* **175**, 564–565 (1972).
12. Tanner, J. T. *Ecology* **56**, 855–867 (1975).
13. Murdoch, W. W. & McCauley, E. *Nature* **316**, 628–630 (1985).

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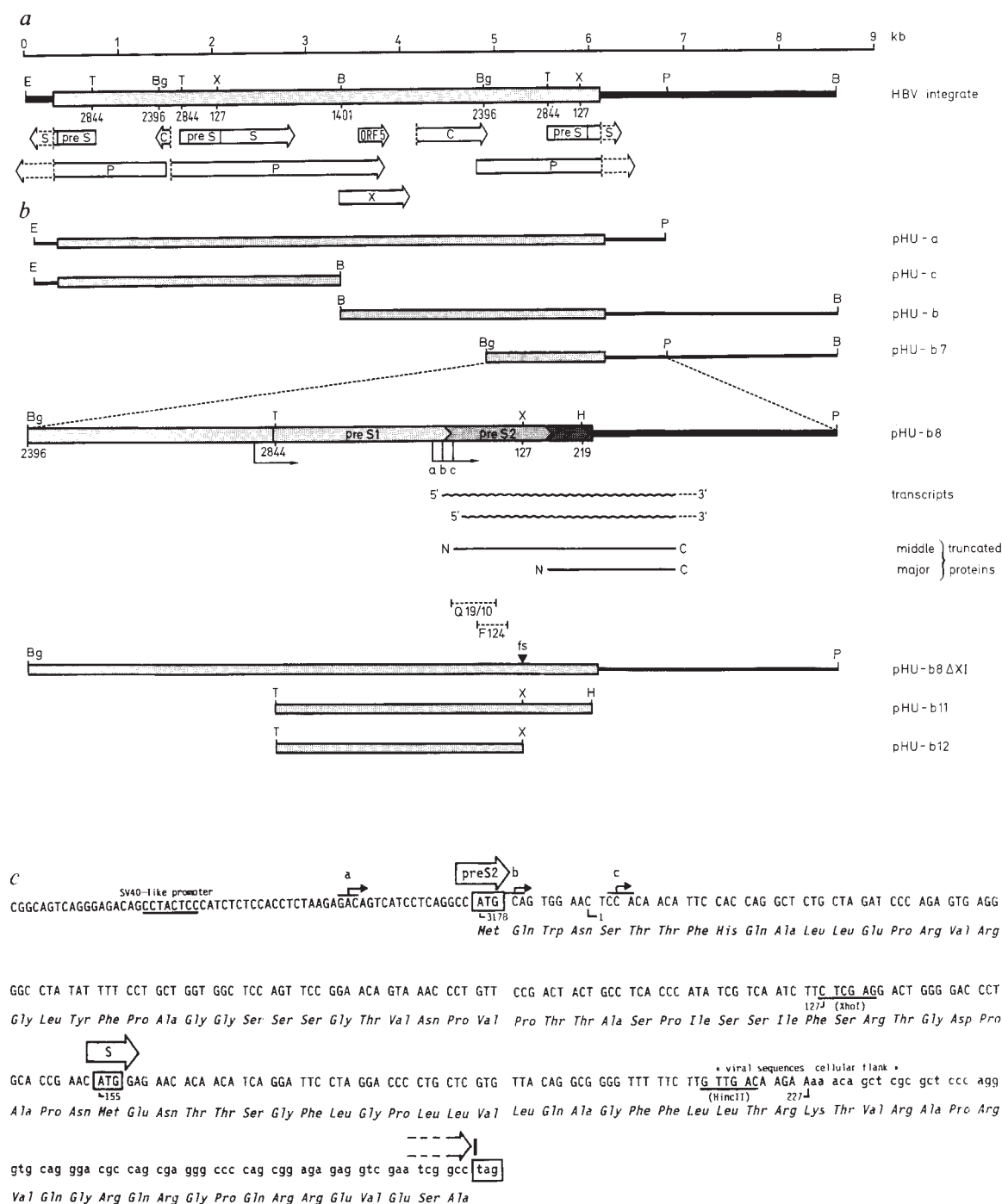
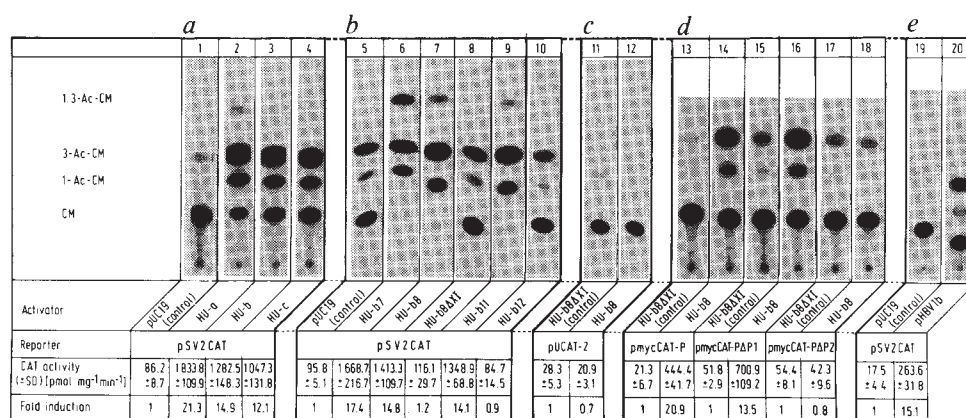


FIG. 1 *a*, Genetic organization of the integrated HBV sequence (subtype adr) from the hepatocellular carcinoma line huH-4, as determined by cloning of integrated HBV DNA into the cosmid vector pHC79 and sequencing of the resulting cosmid clone phu-4/3. Positions of restriction sites (E, *EcoRI*; T, *TaqI*; Bg, *BglII*; X, *XhoI*; B, *BamHI*; P, *PstI*; H, *HincII*) are indicated. The core gene (C) and the polymerase gene (P) have been destroyed by multiple rearrangements, whereas the *orf 5* (ORF 5) and the gene X are still intact. The *preS/S* region is present three times in the integrated HBV DNA; two copies are truncated at their 3' ends. Hatched box, integrated HBV DNA; dark line, cellular flanking sequences. *b*, Identification of the *trans*-activating region by subcloning of the integrated huH-4 HBV DNA (see text). For the *trans*-activating plasmid pHU-b8, the published *preS2* initiation sites (a, b, c), *preS2* transcripts and expected translation products are indicated. Depicted below are the binding sites of the monoclonal antibodies F124 (ref. 30) and Q19/10 (ref. 31), which gave a positive ELISA signal with supernatants from CCL13 cells transfected with pHU-b8. *c*, Sequence analysis of the *trans*-activating truncated *preS2/S* region shows a viral-cellular fusion frame of 102 amino acids. Frameshift mutation or 3' deletion at nucleotide 127 (*XhoI*)

eliminated the *trans*-activating effect completely. The transcription initiation sites (a, b, c), *preS2/S* start codons and the cellular stop codon are indicated. **METHODS.** All constructs were made by using standard recombinant DNA techniques and pUC19 as the vector. Subclones pHU-a, pHU-b and pHU-c were constructed by cutting the respective fragments from the cosmid clone phu-4/3 and ligating them into pUC19; pHU-b7, pHU-b8, pHU-b11 and pHU-b12 were subcloned from pHU-b. Plasmid pHU-b8ΔXI was constructed by digestion of pHU-b8 with *XhoI*, 'blunt-ending' with Klenow enzyme, religation and confirmation of the frameshift by DNA sequencing. For plasmid pHBV1b, the 1.2-kb *RsaI* fragment (nucleotides 2508–573) containing a 3'-truncated *preS/S* sequence was cut out from cloned viral HBV DNA²⁷ and ligated into pUC19. ELISAs were performed as previously described³² by incubating supernatants from CCL13 cells transfected with plasmid pHU-b8 (or pUC19 as a control) in microtitre plates coated with monoclonal antibodies Q19/10 or F124, respectively. Specifically bound PreS2/S protein was detected in a second incubation with peroxidase-labelled anti HBsAg serum (Enzygnost Behring), according to the manufacturer's recommendations.

FIG. 2 Integrated HBV DNA cloned from the huH-4 cell line contains a pSV2CAT-activating function (a); in pSV2CAT, the CAT gene is under the control of the SV40 enhancer-early promoter element²². Also the *c-myc* P2 promoter is activated (d), whereas the enhancerless control plasmid pUCAT-2 shows no induction (c). The activating function can be localized to the 3'-truncated *preS2/S* orf (b). A 3'-truncated viral *preS2/S* sequence likewise activates pSV2CAT (e). CCL13 cells were seeded at $\sim 6 \times 10^5$ cells per 6-cm dish and co-transfected 24 h later with 1 μ g pSV2CAT (or 10 μ g weaker-expressing pmycCAT constructs or pUCAT-2, respectively) and a fivefold molar excess of activator plasmid using the calcium phosphate co-precipitation method³³ with at least three different preparations of CsCl gradient-purified plasmids. Cells were harvested 48 ± 5 h later and the protein concentration in the cell extracts determined by a commercial kit (Bio-Rad). CAT assays were performed as previously described²² with 50–100 μ g protein and the acetylated (Ac-CM) and unreacted [¹⁴C]chloramphenicol (CM) quantified with an isotope scanner (Berthold, FRG). The autoradiograph shown is the result of a typical



sequences in plasmid pHU-b8 and the deletion of all cellular DNA and the *preS1* promoter in plasmid pHU-b11 (Fig. 1b) did not significantly influence the stimulatory effect (Fig. 2b, lanes 7 and 9), whereas an additional 92-base pair (bp) 3' deletion in pHU-b12 (Fig. 1b) caused complete elimination of the *trans*-activation effect (Fig. 2b, lane 10). We conclude that the activating activity is encoded in the *preS/S* region of HBV, and that at least a part of the HBV *preS2/S* sequence deleted in pHU-b12 is necessary for the stimulation.

To investigate if the observed effect was due to a stimulation of *cis*-acting regulatory elements in pSV2CAT, we co-transfected CCL13 cells with pHU-b8 and the reporter plasmid pUCAT2 (ref. 24), which differs from pSV2CAT in that it lacks the simian virus 40 (SV40) enhancer. No stimulation was observed under these conditions (Fig. 2c). The *trans*-activation of pSV2CAT is therefore dependent on the presence of the SV40 enhancer.

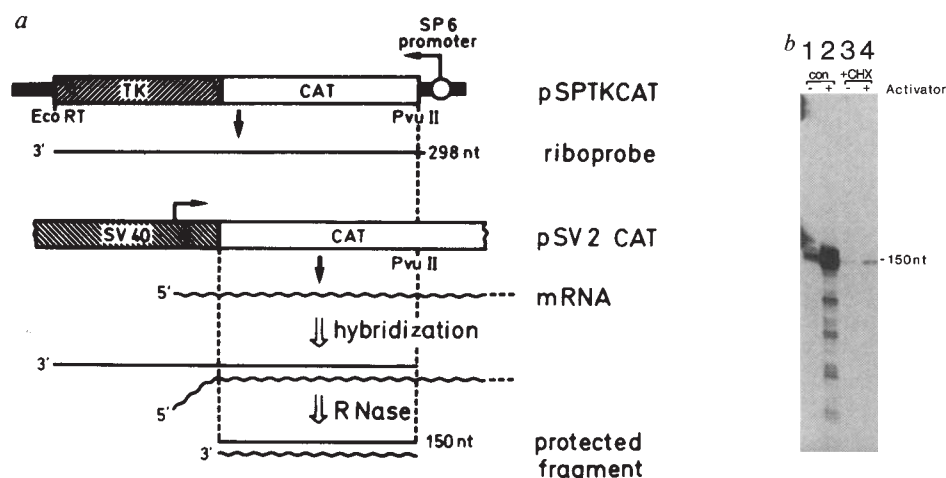
This result indicates that the stimulation of CAT expression

set of experiments. CAT activities were calculated as mean values (and s.d.s) from three independent transfections, in pmol acetylated chloramphenicol per minute and mg protein. Induction was calculated as the ratio of the stimulated to the control values (control) of the respective experiments. In b (lanes 6, 7, 9), the given values might be underestimations of the actual inductions, owing to almost expired substrate.

occurs at the level of transcription; to prove this we co-transfected CCL13 cells with pSV2CAT and pHU-b8 and measured CAT-specific transcripts in a quantitative RNase protection assay. Plasmid pHU-b8 caused an increase of the 150-nucleotide messenger RNA fragment from plasmid pSV2CAT (11:1 as determined by densitometric scanning; Fig. 3b, lanes 1 and 2), which is within the same range as the activation in simultaneously performed CAT assays (15:1, data not shown). The stimulatory effect of the integrated HBV *preS/S* sequence on pSV2CAT is therefore due to transcriptional *trans*-activation.

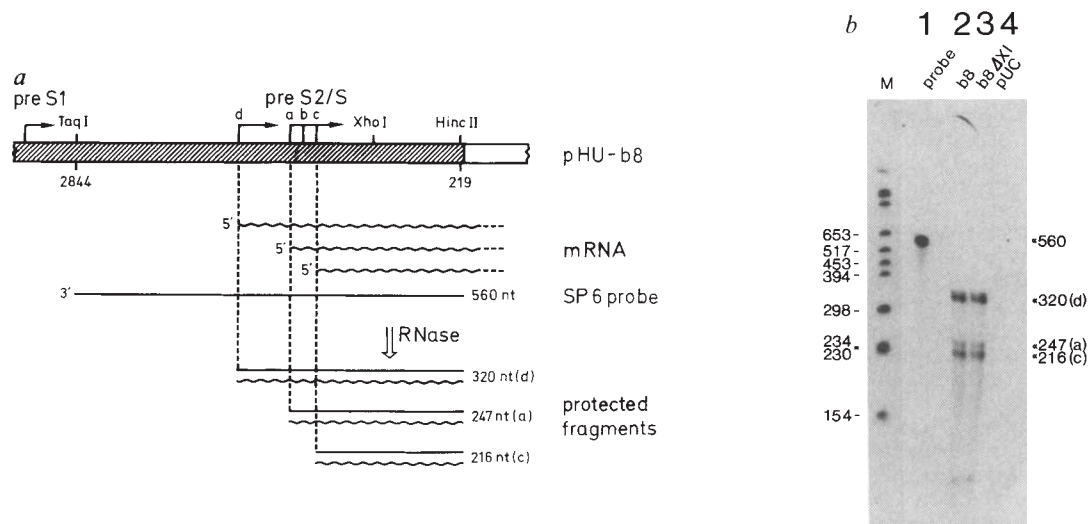
Two different promoter elements are known to be involved in the transcription of the *preS/S* genes: a TATA-sequence box upstream of the *preS1* start codon, and a sequence with homologies to the SV40 origin-late promoter, which begins ~ 70 bp upstream of the *preS2* translation start site^{2,25,26}. Because plasmid pHU-b11, which still encodes a *trans*-activating activity, is lacking the TATA-like *preS1* promoter, the transactivator

FIG. 3 The activation of pSV2CAT by the *preS2/S*-encoded transactivator occurs at the transcriptional level and can be eliminated by cycloheximide. Quantitative RNase protection assay of CAT-specific mRNA after co-transfection of pSV2CAT and the *trans*-activating plasmid pHU-b8 (+) or pUC19 as a control (–), in the presence (CHX) or absence (con) of cycloheximide. Nucleotide, nt. a, Structure of the riboprobe, the pSV2CAT transcript and the protected fragment. The riboprobe vector pSPTKCAT³⁴ contains the first 150 bp of the CAT gene fused to the herpes simplex virus thymidine kinase (TK) gene promoter and generates a 298-nucleotide probe after digestion with *Eco*RI and *in vitro* transcription with SP6 polymerase. Hybridization to the 5' part of the transcript from pSV2CAT protects a 150-nucleotide RNase-resistant fragment, corresponding to the identical sequence between the riboprobe and the pSV2CAT mRNA. b, Quantitative RNase protection assay shows an increase (11:1, as determined by densitometric scanning) of the 150-nucleotide CAT-specific signal after co-transfection of pSV2CAT with the activator plasmid pHU-b8 (lane 2) as compared with pUC19 as a control (lane 1). Addition of 30 μ g ml⁻¹ cycloheximide to the medium 3 min before transfection (lane 3, pSV2CAT and pUC19; lane 4, pSV2CAT and pHU-b8) eliminates the stimulatory effect (1.4:1).



METHODS. CCL13 cells were seeded at $\sim 10^6$ cells per 10-cm dish and co-transfected 24 h later with 2 μ g pSV2CAT and a fivefold molar excess of pHU-b8 or pUC19, respectively. Total cellular RNA was isolated as described³⁵ 12–18 h later, and treated with RNase-free DNase (Promega) to remove contaminating plasmid DNA. Aliquots of 20 μ g RNA were analysed by quantitative RNase protection as previously described³⁴. Standard CAT assays were performed in parallel 48 ± 5 h after transfection.

FIG. 4 The *pres2/S* transactivating plasmid pHU-b8 and its frameshift mutant pHU-b8ΔXI are correctly transcribed after transfection of CCL13 cells. *a*, Structure of the pHU-b8 transcription unit. Transcripts from the *pres2* initiation sites *a*, *c* and *d* protect RNase-resistant fragments of 320, 247 or 216 nucleotides (nt) after hybridization with the 560-nucleotide (*TaqI*-*HincII*) riboprobe. To generate this autologous riboprobe, the 560-bp *TaqI*-*HincII* insert of pHU-b11 was ligated into the multiple cloning site of pGEM-2 (Promega) and the resulting vector pGEM-b11 transcribed with SP6 after *HindIII* digestion. *b*, RNase protection assays of



a and c of the SV40-like *preS2* promoter²⁶. The signal d of 320 nucleotides would correspond to an additional transcript starting at HBV nucleotide 3,088±20, at the 5'-most end of the SV40-like region²⁵; whether this reflects a conserved initiation site of general significance is now under investigation.

mRNA can be assumed to be transcribed from the SV40-like *preS2* promoter region. To show the actual transcription of the *preS2/S* region, we performed an RNase protection assay with RNA prepared from CCL13 cells transfected with the *trans*-activating plasmid pHU-b8 (Fig. 4b, lane 2). All three protected bands of 320, 247 and 216 nucleotides correspond to *preS2/S*-specific transcripts, whereas no transcripts from the *preS1* promoter were detected.

In the transcribed region, we identified a viral-cellular fusion *Orf* of 102 amino acids, encoding a PreS/S ('middle S'²) protein which is truncated at a position corresponding to HBV nucleotide 227 (Fig. 1c). We confirmed the expression of this *orf* from the *trans*-activating plasmid pHU-b8 by enzyme-linked immunosorbent assays (ELISAs), using monoclonal antibodies against PreS2 epitopes (Fig. 1b). To test whether this *orf* is responsible for the observed *trans*-activation, we introduced a (-1) frameshift mutation in the *preS2 orf* (pHU-b8ΔX1, Fig. 1b). Because the *trans*-activating effect was completely eliminated by this frameshift mutation (Fig. 2b, lane 8), and the analysis of mRNA showed that there was no impairment of transcription (Fig. 4b, lane 3), the transactivator is likely to be a translation product of the truncated *preS2/S orf*. In this case, the stimulation should be suppressible by inhibitors of protein synthesis. So we co-transfected CCL13 cells with pHU-b8 and pSV2CAT in the presence of 30 µg ml⁻¹ cycloheximide and measured the amount of CAT-specific mRNA by a quantitative RNase protection assay. The resulting elimination of the stimulatory effect (Fig. 3b, compare lanes 1 and 2 with lanes 3 and 4) indicates that the *trans*-activation requires *de novo* protein synthesis.

To find out whether truncated *preS2/S* sequences of the wild-type virus genome also possess a *trans*-activation potential, we constructed the plasmid pHBV1b, which contains an artificially 3'-truncated *preS2/S* gene from cloned viral HBV DNA²⁷ (see legend to Fig. 1 for details). Plasmid pHBV1b likewise led to a significant increase in CAT activity in co-transfection experiments (Fig. 2e).

By considering these results together, it can be concluded that a protein synthesized from 3'-truncated *preS2/S* sequences of integrated or genomic HBV DNA can act as a transcriptional transactivator. The full-length *preS2/S* gene did not show any

trans-activation activity in co-transfection experiments (refs 19 and 28, and our unpublished observations). From this observation we hypothesize that rearrangements during viral DNA integration lead to the generation of C-terminally truncated *preS2/S* gene products, which have *trans*-activation activity as a consequence of altered protein function or intracellular distribution, respectively.

Because the cellular oncogene *c-myc* is strongly expressed in the huH-4 cell line (our unpublished data), we co-transfected CCL13 cells with pHU-b8 and the reporter plasmid pmycCAT-P, which contains the *CAT* gene under control of the human dual *c-myc* promoter²⁹. As controls, plasmids pmycCAT- Δ P1 or pmycCAT- Δ P2 were used, in which the first or the second element of the dual promoter are deleted, respectively²⁹. Both pmycCAT-P and pmycCAT- Δ P1 were inducible by co-transfected plasmid pHU-b8, whereas deletion of the *c-myc* P2 promoter in pmycCAT- Δ P2 abolished the effect completely (Fig. 2d). These results indicate that 3'-truncated *preS2/S* sequences from integrated HBV DNA can stimulate the cellular *myc* P2 promoter, suggesting a possible mechanism for liver carcinogenesis by activation of cellular genes *in trans*.

The *preS2/S* region is integrated in nearly all hepadnavirus-related human and animal HCCs analysed so far²⁻¹⁷; in many cases at least one copy of the *preS2/S* gene is truncated at its 3' end⁸⁻¹⁷. Also, a second integrated 3'-truncated *preS/S* sequence (nucleotides 2703-217) cloned directly from human hepatoma tissue, yields a similar set of data in pSV2CAT cotransfection experiments (data not shown). Together with the results presented here, these findings support the idea that accidental 3' truncations of integrated HBV *preS2/S* genes could be a causative factor in HBV-associated oncogenesis. □

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1. Beasley, R. P. & Hwang, L. Y. in *Viral Hepatitis and Liver Disease* (eds Vyas, G. N., Dienstag, J. L. & Hoofnagle, J. H.) 209-214 (Grune & Stratton, New York, 1984).
2. Ganem, D. & Varmus, H. E. *A. Rev. Biochem.* **56**, 651-693 (1987).
3. Bréchot, C., Pourcel, C., Louise, A., Rain, B. & Tiollais, P. *Nature* **286**, 533-535 (1980).
4. Dejean, A., Bougueleret, L., Grzeschik, K.-H. & Tiollais, P. *Nature* **322**, 70-72 (1986).
5. Edman, J. C., Gray, P., Valenzuela, P., Rall, L. B. & Rutter, W. J. *Nature* **286**, 535-538 (1980).
6. Koshy, R. et al. *Cell* **34**, 215-223 (1983).
7. Yaginuma, K., Kobayashi, M., Yoshida, E. & Koike, K. *Proc. natn. Acad. Sci. U.S.A.* **82**, 4458-4462 (1985).
8. Berger, I. & Shaul, Y. *J. Virol.* **61**, 1180-1186 (1987).

9. Choo, K.-B. *et al.* *Virology* **154**, 405-408 (1986).
10. Fowler, M. J., Thomas, H. C. & Monjardino, J. *J. Virol.* **67**, 771-775 (1986).
11. Imai, M. *et al.* *J. Virol.* **61**, 3555-3560 (1987).
12. Kolke, K. *et al.* *Nucleic Acids Res.* **11**, 5391-5402 (1983).
13. Nagaya, T. *et al.* *Genes Dev.* **1**, 773-782 (1987).
14. Ogston, C. W., Jonak, G. J., Rogler, C. E., Astrin, S. M. & Summers, J. *Cell* **29**, 385-394 (1982).
15. Shaul, Y., Garcia, P. D., Schonberg, S. & Rutter, W. J. *J. Virol.* **59**, 731-734 (1986).
16. Tur-Kaspa, R., Burk, R. D., Lieberman, H. H. & Shafritz, D. A. *J. Hepat.* **3**, S25-S33 (1986).
17. Ziemer, M., Garcia, P., Shaul, Y. & Rutter, W. J. *J. Virol.* **53**, 885-892 (1985).
18. Wollersheim, M., Debelka, U. & Hofschneider, P.-H. *Oncogene* **3**, 545-554 (1988).
19. Twu, J.-S. & Schloemer, R. H. *J. Virol.* **61**, 3448-3453 (1987).
20. Zahm, P., Hofschneider, P. H. & Koshy, R. *Oncogene* **3**, 169-177 (1988).
21. Huh, N. & Utakoji, T. *Gann* **72**, 178-179 (1981).
22. Gorman, C. M., Moffat, L. F. & Howard, B. H. *Molec. cell. Biol.* **2**, 1044-1051 (1982).
23. Kaneko, S. & Miller, R. J. *J. Virol.* **11**, 3979-3984 (1988).
24. Haslinger, A. & Karin, M. *Proc. natn. Acad. Sci. U.S.A.* **82**, 8572-8576 (1985).
25. Cattaneo, R., Will, H., Hernandez, N. & Schaller, H. *Nature* **305**, 336-338 (1983).
26. Standing, D. N., Rutter, W. J., Varmus, H. E. & Ganem, D. *J. Virol.* **50**, 563-571 (1984).
27. Will, H. *et al.* *Proc. natn. Acad. Sci. U.S.A.* **82**, 891-895 (1985).
28. Seto, E., Yen, T. B., Peterlin, B. M. & Ou, J.-H. *Proc. natn. Acad. Sci. U.S.A.* **85**, 8286-8290 (1988).
29. Lipp, M., Schilling, R., Wiest, S., Laux, G. & Bornkamm, G. W. *Molec. cell. Biol.* **7**, 1393-1400 (1987).
30. Budkowska, A., Dubreuil, P., Riottot, M.-M., Briantais, M.-J. & Pillot, J. *J. Immunol. Meth.* **97**, 77-85 (1987).
31. Heermann, K.-H., Waldeck, F. & Gerlich, W. H. in *Viral Hepatitis and Liver Disease* (ed. Zuckermann, A. J.) 697-700 (Liss, New York, 1988).
32. Marquardt, O., Heermann, K.-H., Seifer, M. & Gerlich, W. H. *Archs Virol.* **96**, 249-256 (1987).
33. Graham, F. L. & van der Eb, A. J. *Virology* **52**, 456-467 (1973).
34. Angel, P. *et al.* *Cell* **49**, 729-739 (1987).
35. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. *Biochemistry* **18**, 5294-5299 (1979).

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Inhibition of neurite polarity by tau antisense oligonucleotides in primary cerebellar neurons

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NEURONS in culture can have fundamentally distinct morphologies which permit their cytological identification and the recognition of their neurites as axons or dendrites. Microtubules may have a role in determining morphology by the selective stabilization of spatially distinct microtubule subsets. The plasticity of a neurite correlates inversely with the stability of its component microtubules: microtubules in growth cones are very dynamic, and in initial neurites there is continuous incorporation of labelled subunits¹, whereas in mature neurites, microtubules are highly stabilized². The binding of microtubule-associated proteins to the microtubules very probably contributes to this stability. Cerebellar neurons in dissociated culture initially extend exploratory neurites and, after a relatively constant interval, become polarized⁴. Polarity becomes evident when a single neurite exceeds the others in length. These stable neurites cease to undergo the retractions and extensions characteristic of initial neurites and assume many features of axons and dendrites. We have now studied the role of the neuronal microtubule-associated protein tau in neurite polarization by selectively inhibiting tau expression by the addition of antisense oligonucleotides to the culture media. Although the extension of initial exploratory neurites occurred normally, neurite asymmetry was inhibited by the failure to elaborate an axon.

Primary neurons from embryonic day-15 primordial rat cerebella were dissociated and plated on polylysine-coated coverslips⁴. After 1 h in serum-containing media they were changed to serum-free defined media⁴ containing one of the various antisense or sense oligonucleotides, complementary to

5' sequence of the tau gene (Fig. 1). We have shown by mass spectrometry of bovine brain tau that the AUG predicted from the tau complementary DNA sequence specifies an initiator methionine *in vivo* (K.S.K. and H. Scoble, unpublished data). The addition of 50 μ M antisense oligonucleotide RT11-14 resulted in a strong inhibition of neurite elongation (Table 1). After 24 h, sense-treated neurons contained a single neurite that exceeded the others in length and assumed the appearance of an axon (Fig. 2). At identical time points the antisense-treated neurons contained short neurites without a single elongated process. The reduction in total neurite length was solely due to the inhibition of this axon-like neurite, because there was no change in neurite length in any of the other minor neurites (Fig. 3a). The absolute numbers of neurites between the sense- and antisense-treated cultures did not differ. By 72 h, without any change in the media, antisense-treated cultures began to recover polarity, but continued to lag in total neurite length. The completely non-overlapping oligonucleotides, designated RT23 and RT24 (Fig. 1), had identical effects. Oligonucleotide RT20 was less effective in inhibiting neurite elongation (Table 1), probably because of differences in the secondary structure of the RNA. Sense controls for each oligonucleotide had no effect on the cultures. A dosage effect was demonstrated by a graded release of inhibition with a reduction in the concentration of the antisense oligonucleotide (Table 1).

The specific effect of antisense oligonucleotides on the expression of tau protein was demonstrated immunohistochemically with the anti-tau monoclonal antibody 5E2 (ref. 5). In both untreated cultures and sense-treated cultures, trace amounts of tau-immunoreactivity was observed after 12 h. By 24 h, tau-immunoreactivity was evident and appeared to segregate within the axon-like neurite (Fig. 4). In antisense-treated cultures, only trace amounts of tau-immunoreactivity were observed during this time period, and the more symmetrical appearance of the neurites precludes its segregation. In agreement with the cell-staining result, immunoblots revealed the immature tau doublet in the sense-treated cells and only trace reactivity in the antisense-treated cells. To quantitate this effect, dot immunobinding¹¹ with two different monoclonal antibodies (5E2 and tau 1; ref. 5) after 24 h in culture demonstrated non-detectable levels of tau in the antisense-treated cultures, and 100 ng tau-immunoreactive protein per mg total cellular protein in the sense-treated cultures (Fig. 3b). By contrast, there was abundant staining with anti-tubulin antibodies (Tu9B and J1) under both sense and antisense conditions (Fig. 2). Dot immunobinding with the tubulin monoclonal antibodies confirmed the lack of any significant difference between the sense and antisense treatments for tubulin concentration. MAP2 reactivity with monoclonal antibody 5F9 (ref. 12) in these cells was also intense and unaffected by antisense treatment (data not shown). When the cells entered a recovery phase as judged by the presence of a

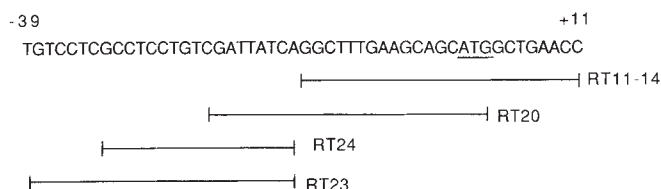


FIG. 1 Tau cDNA sequence with the ATG codon that corresponds to the initiator methionine underlined. Each of the antisense synthetic oligonucleotides (RT11-14, RT20, RT24 and RT23) are the inverse complements of the regions indicated. The sense oligonucleotides (RT18, RT19, RT26 and RT25, respectively) are the exact inverse complements of the antisense oligonucleotides. All oligonucleotides were synthesized on an Applied Biosystems 380B synthesizer, purified over a NAP 5 column (Pharmacia), ethanol precipitated, and taken up in media.

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TABLE 1 Antisense inhibition (dosage effect)

Oligonucleotide	Time in culture (h)	Concentration (μM)	Neurite length (μM)	Neurite no.
Sense (RT18)	24	50	150.79 $\pm$ 8.2	3.67 $\pm$ 0.42
Antisense (RT11-14)	24	50	36.66 $\pm$ 2.4	3.10 $\pm$ 0.34
	24	25	45.44 $\pm$ 3.5	3.10 $\pm$ 0.26
	24	12.5	71.55 $\pm$ 5.7	3.25 $\pm$ 0.24
	24	6.25	101.1 $\pm$ 12.5	3.65 $\pm$ 0.20
	24	3.12	136.2 $\pm$ 6.4	3.44 $\pm$ 0.15
	72	50	139.25 $\pm$ 4.8	3.10 $\pm$ 0.20
	72	50	139.25 $\pm$ 4.8	3.10 $\pm$ 0.20
Antisense (RT20)	24	25	84.64 $\pm$ 6.4	3.38 $\pm$ 0.16
Sense (RT19)	24	25	140.2 $\pm$ 8.4	3.48 $\pm$ 0.08
No treatment	24	0	155.9 $\pm$ 12.9	3.54 $\pm$ 0.20
	72	0	460.0 $\pm$ 15.1	3.64 $\pm$ 0.35

At dosages of 12.5 μM and higher, 50 cells were analysed; at dosages of 6.25 and lower, 25 cells were analysed.

single elongated neurite, tau-immunoreactivity was partitioned to this axon-like compartment (Fig. 4).

Although the effects of these oligonucleotides were readily observable, an attempt was made to demonstrate that the oligonucleotides entered the cell. Antisense RT11-14 (4 μg) was end-labelled with [γ - ^{32}P]ATP and T4 polynucleotide kinase¹³, purified by polyacrylamide gel electrophoresis¹⁴, and the eluted sample ethanol-precipitated and resuspended in media. An activity of 2.5×10^6 c.p.m. was added to the culture media

without unlabelled oligonucleotide so as not to saturate uptake. After 1 h and 3 h, the neurons were scraped and the activity determined in the media, the cytosol and the nuclear fraction. After 1 h, 0.5% of the total activity was cytosolic and 0.01% was nuclear. After 3 h, 2.5% of the total activity was cytosolic and 0.1% was nuclear. Although a small, but probably significant, amount of radiolabel was able to enter the cell, whether the oligonucleotide remained intact after passage through the plasmalemma or whether its presence in the cell represented entry of single nucleotides or exchanged ^{32}P was determined. An acrylamide gel of the cytosol at the same time intervals revealed that nearly all of the labelled oligonucleotide inside the cell co-migrated with the original oligonucleotide.

Antisense oligonucleotides can be taken up by cells in a saturable size-dependent way compatible with receptor-mediated endocytosis¹⁵. A small percentage of the intact antisense oligonucleotide was able to cross the plasmalemma and affect neurite length in a way unrelated to the overall vitality of the cells. The efficacy of the strategy here could be due to tau mRNA becoming expressed in the cerebellar macroneurons at the time they are plated. Thus in the presence of a low copy number of tau mRNAs, a small amount of nucleotide could be effective in reducing tau expression. The onset of transcription could provide a vulnerable period during which antisense works. Furthermore, the configuration of tau in embryonic cerebellum does not show the multiple complex isoforms seen later on; it is a doublet of relative molecular mass 52,000 (ref. 8) and therefore could have a single 5' untranslated sequence. After 72 h the cells recovered polarity, but the mean summed length of their neurites continued to lag behind that of controls. This interval to recovery approximates the plateau at 50 h in the intracellular accumulation of acridine-labelled oligonucleotide¹⁵.

Although no effect was observed on the intensity with which the cultures were labelled by anti-tubulin antibody, tau-immunoreactivity remained at trace levels until 36 h after plating. Tau functions to stabilize microtubules⁷; so tubulin synthesis in excess of tau, relative to control conditions, could lead to persistent unstable microtubules, characteristic of filopodia, and

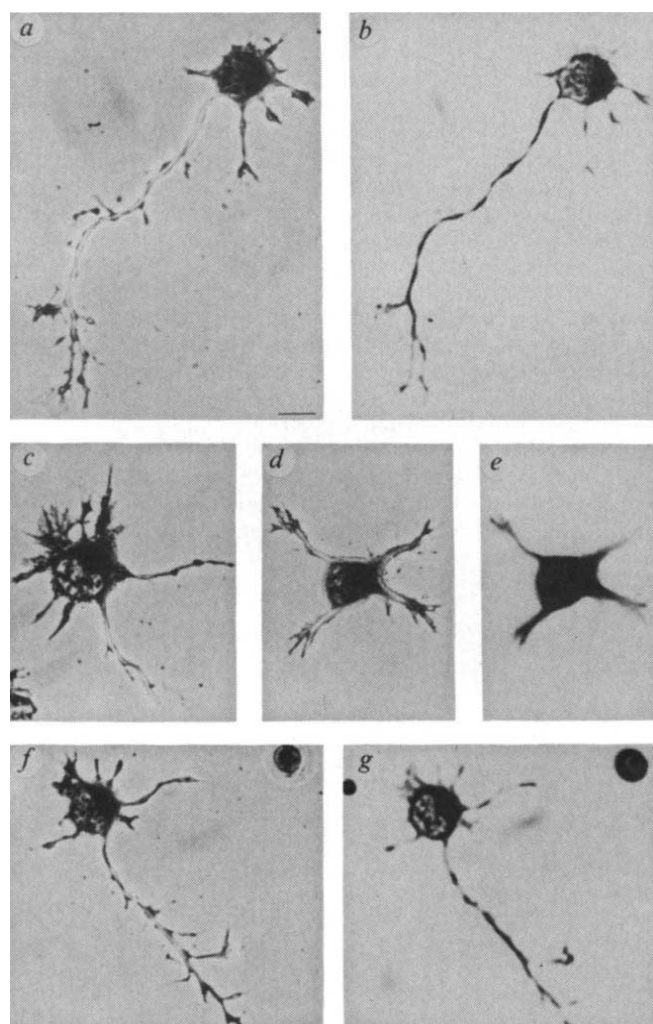


FIG. 2 Cerebellar neurons in culture. *a*, Phase micrograph after 24-h treatment with sense oligonucleotide RT18. *b*, Anti-tubulin-antibody staining of the same cell as in *a*. Staining at the stage shown here and subsequently was done with the mouse Extravidin kit (Sigma) after methanol fixation. *c*, *d*, Phase micrograph after 24-h treatment with antisense oligonucleotide RT11-14. These cells failed to become polarized and in some cases still contained lamellipodia (*c*). *e*, Anti-tubulin antibody staining of the same cell as in *d* indicates presence of tubulin in this cell. *f*, Phase micrograph after 72-h treatment with antisense oligonucleotide RT11-14 demonstrates recovery of polarity. *g*, Anti-tubulin antibody staining of the same cell as in *f*. Scale bar, 10 μm .

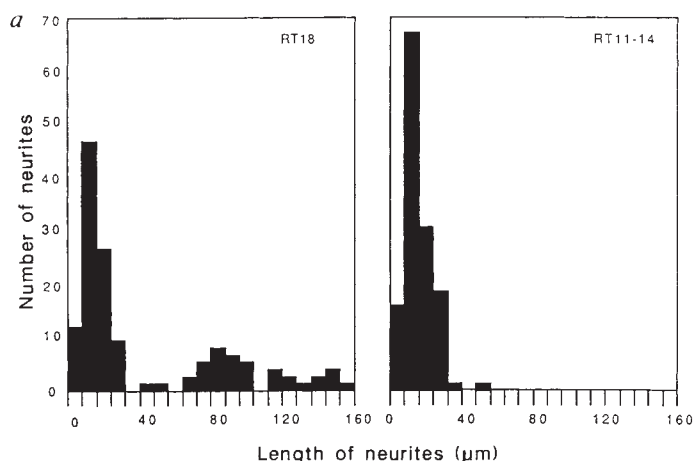
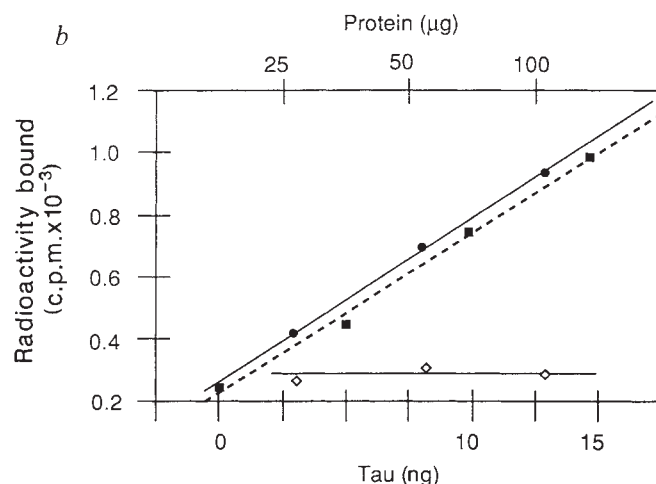


FIG. 3 *a*, Bar chart showing inhibition of polarity. *b*, Dot immunobinding to quantitate the inhibition of tau expression after antisense treatment. The levels of tau protein were determined by assay with ^{125}I -labelled protein A, and here, the anti-tau monoclonal antibody 5E2. The mass standard (■) is purified tau protein. Standard curves were constructed and the amount of



tau in the high-speed supernatant of the unknown samples was determined by extrapolation of the linear regions of the unknown to the linear portions of the standard. ●, Sense-treated; ◇, anti-sense treated. Background levels were 250 c.p.m.

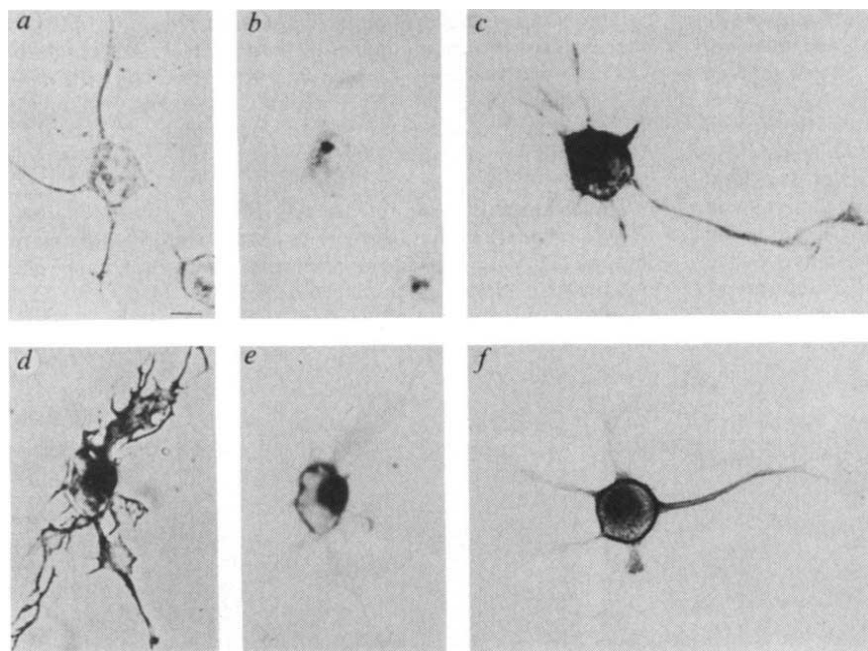


FIG. 4 Cerebellar neurons in culture. *a*, Phase micrograph after 24-h treatment with antisense oligonucleotide RT11-14. *b*, Bright-field view of the same cell as in *a* stained with anti-tau antibody indicates negligible reaction product. *c*, Treatment (24 h) with sense oligonucleotide and stained with anti-tau antibody demonstrates the presence of immunoreactivity in the soma and the axon-like compartment. *d*, Phase micrograph after 50-h treatment with antisense demonstrates continued inhibition of polarity. *e*, Bright-field view of the same cell as in *d* stained with anti-tau antibody has negligible reaction product. *f*, Treatment (72 h) with antisense oligonucleotide stained with anti-tau antibody during recovery of polarity. The length and configuration of the neurites present at this time interval are similar to those observed after only 24 h in the sense-treated cultures shown in *c*. Scale bar, 10 μm .

inability to achieve the crucial length associated with polarization⁶. Mitchison and Kirschner³ proposed three phases of axonal development: an actin-based system in which the leading edge becomes orientated, a consolidation phase in which filopodial microtubules become stabilized in their direction of future growth, and a conversion phase to stable microtubules bundled within the axonal tube. Tau does not seem to be required for early exploratory neurites. The data presented here indicate that tau-transcript antisense oligonucleotides inhibit the conversion phase, and thereby prevents the formation of a permanent neurite ready to assume its identity as an axon. Thus inhibition of tau is sufficient to inhibit polarity, but expression of tau is insufficient to induce polarity⁹. Finally, the inhibition induced by antisense oligonucleotides could serve as a basis for strategies to reduce the abundant tau-immunoreactive neurites observed in Alzheimer's disease¹⁰. □

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- Okabe, S. & Hirokawa, N. *J. Cell Biol.* **107**, 651-664 (1988).
- Horio, T. & Hotani, H. *Nature* **321**, 605-607 (1986).
- Mitchison, T. & Kirschner, M. *Neuron* **1**, 761-772 (1988).
- Ferreira, A., Busciglio, J. & Caceres, A. *Devl Brain Res.* **34**, 9-31 (1987).
- Kosik, K. S. et al. *Neuron* **1**, 817-825 (1988).
- Goslin, K. & Banker, G. *J. Cell Biol.* **108**, 1507-1516 (1989).
- Drubin, D. G. & Kirschner, M. W. *J. Cell Biol.* **103**, 2739-2746 (1986).
- Ferreira, A., Busciglio, J. & Caceres, A. *Devl Brain Res.* **49**, 215-228 (1989).
- Kosik, K. S. & Finch, E. A. *J. Neurosci.* **7**, 3142-3153 (1987).
- Kowall, N. W. & Kosik, K. S. *Ann. Neurol.* **22**, 639-643 (1987).
- Jahn, R., Schiebler, W. & Greengard, P. *Proc. natn. Acad. Sci. U.S.A.* **81**, 1684-1687 (1984).
- Kosik, K. S., Orecchio, L. D., Bakalis, S., Duffy, L. & Neve, R. L. *J. Neurochem.* **51**, 587-598 (1988).
- Tabor, S. in *Current Protocols in Molecular Biology* Vol. 1 (eds Ausubel, F. B. et al.) 3.10.4-3.10.5 (Wiley, New York, 1989).
- Chorey, J. in *Current Protocols in Molecular Biology* Vol. 1 (eds Ausubel, F. B. et al.) 2.7.1-2.7.5 (Wiley, New York, 1989).
- Loke, S. L. et al. *Proc. natn. Acad. Sci. U.S.A.* **86**, 3474-3478 (1989).

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A threshold effect of the major isoforms of NCAM on neurite outgrowth

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INTERACTIONS between recognition molecules on the surface of neuronal growth cones and guidance cues present in the local cellular environment are thought to account for the growth of neurites in the highly stereospecific manner that contributes to correct target cell innervation¹⁻³. *In vitro* assays have been used to identify candidate molecular components of this system, either directly by demonstrating their ability to promote neurite outgrowth^{4,5}, or indirectly by the ability of specific antibodies to inhibit neurite outgrowth⁶⁻¹⁰. The role of the neural cell adhesion molecule (NCAM)^{2,11} in pathway finding is not fully understood. Some immunological studies support a positive role^{6,8,9}; others do not^{7,10}, and it has been reported that purified NCAM does not support neurite outgrowth⁴. We have previously shown that an arbitrary biochemical index of neurite outgrowth, the relative level of immunoreactive neurofilament protein, is increased when human and rat dorsal root ganglion neurons are cultured on monolayers of cells expressing transfected human NCAM¹². But, the complexity of growth precluded a simple morphological analysis and we did not determine the 'dose-response' relationship between NCAM expression and neuronal response. Here, we report on the morphology of rat cerebellar neurons cultured on monolayers of 3T3 cells transfected with complementary DNAs encoding all of the main NCAM isoforms found in cells such as astrocytes,

Schwann cells and skeletal muscle¹³. The data indicate that both transmembrane and glycosyl-phosphatidylinositol linked NCAM isoforms are potent substrates for neurite extension. A critical threshold value of NCAM expression is required for increased neurite outgrowth. Above this threshold, small increases in NCAM induce substantial increases in neurite outgrowth.

The preparation and biochemical characterization of the 3T3 cell clones transfected with cDNAs corresponding to the major NCAM isoforms found in non-neuronal cells have been described in detail elsewhere^{12,14-17}. Here, we have cultured dissociated neurons from postnatal day 7 rat cerebellum on confluent monolayers of 3T3 cells expressing one of four NCAM isoforms. These were the transmembrane isoform (relative molecular mass 140,000 (140K)), the 120K glycosyl-phosphatidylinositol (GPI)-linked isoform and a 125K muscle-specific variant of the latter^{15,16}. As one control, we cultured neurons on monolayers of cells transfected with a cDNA encoding a secreted NCAM isoform¹⁴. After 20-21 h, the co-cultures were carefully fixed and labelled with a monoclonal antibody that binds to polysialic acid residues present on neuronal NCAM, but not the human NCAM synthesized by 3T3 cells; both this antibody and a second, independent rabbit anti-serum that binds to all the NCAM isoforms labelled all of the dissociated cerebellar cells. Co-cultures were scanned in a systematic manner and all labelled cells with one or more processes of more than 15 μ m were recorded on video tape for subsequent computer-assisted morphometric analysis. The morphology of neurons grown on monolayers of both control and transfected 3T3 cells was relatively simple, with approximately 90% of the cells having fewer than three primary processes (Fig. 1). All process-bearing cells expressed the neurofilament protein, as determined by an immunocytochemical analysis (data not shown).

The total neuritic output per cell (the sum of the lengths of individual neurites) has been plotted as a function of the relative level of NCAM expression for neurons grown on a panel of 11 independent clones of transfected 3T3 cells (Fig. 2a). These included two clones expressing the 140K transmembrane isoform (D1, 5), six clones expressing the 125K muscle-specific

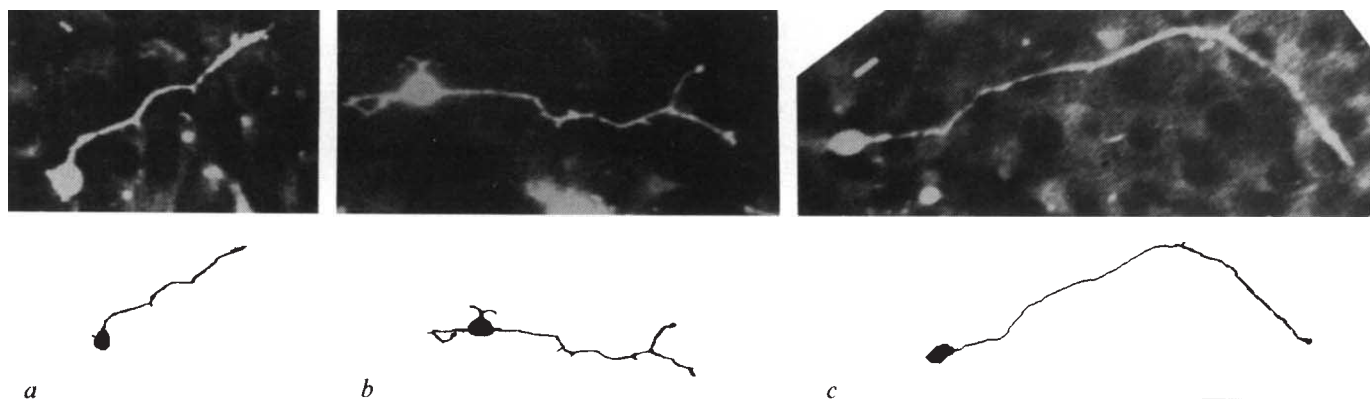
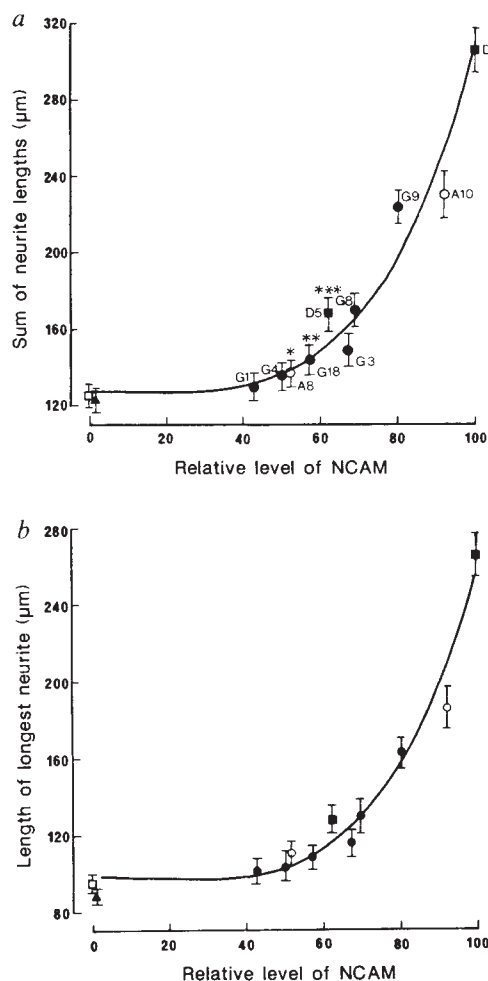


FIG. 1 Morphology of cerebellar neurons on monolayers of *a*, control 3T3 cells or *b*, 3T3 cell transfectant clone D1 or *c*, clone G9. The photographs show the image as directly recorded on videotape. The images as digitized on the video monitor for morphometric analysis are shown underneath. Scale bar, 20 μ m.

METHODS. Small pieces of tissue were dissected from post-natal day 7 rat cerebellum and incubated in Versene solution (Gibco Laboratories) containing trypsin (0.05%, w/v) for 10 min at 37 °C before being dissociated to a single-cell suspension by gentle trituration. Confluent monolayers of control and transfected 3T3 cells were established over a 24-48 h period in individual chambers of a collagen-coated tissue culture slide (Lab-Tek) as described previously¹². Co-cultures were established by adding ~3,000 cerebellar neurons in 200 μ l SATO media to monolayer cultures in chambers

containing 200 μ l Dulbecco's modified Eagles' medium (DMEM)/10% fetal calf serum. After 20-21 h, the co-cultures were fixed with paraformaldehyde and the neurons labelled with a monoclonal antibody to meningococcal type B polysialic acid (Wellcome PLC) that recognizes the α -2,8 linkage of polysialic acid chains present in neuronal NCAM isoforms^{27,28} followed by a rhodamine-conjugated sheep anti-mouse immunoglobulin, essentially as described previously¹². Labelled cells were detected by fluorescence microscopy using an image intensification video camera (Panasonic, model 1900) attached to an inverted Zeiss microscope (ICM 405). The video camera was connected to a video tape recorder (VTR, Sony, V-matic, 5080) and an image analysis system (Sight Systems). This system allows for direct tracing of the image on the video monitor, and for subsequent digitized analysis of the lengths of individual neurites.



GPI-linked isoform (G1, 3, 4, 8, 9, 18), two clones expressing the 120K GPI-linked isoform (A8, 10) and one clone expressing the secreted isoform. Neurite outgrowth on the clone expressing the highest level of NCAM (D1) was almost 2.5 times greater than that found on control monolayers. A similar increase was found if the length of the single longest neurite per cell was measured (Fig. 2b). In contrast, differentiation was unaffected for neurons grown on monolayers of cells that synthesized the secreted NCAM isoform. There is a clear relationship between the level of NCAM expression and the magnitude of response. None of the data points, irrespective of isoform type, deviate substantially from a simple curve. Thus, the chief determinant

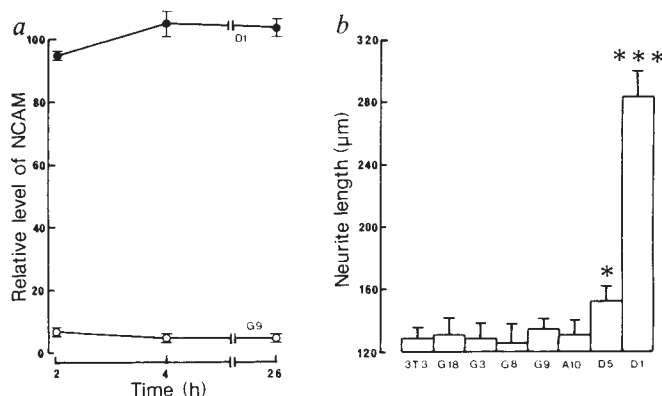
FIG. 2 The relationship between neurite outgrowth and NCAM expression at the cell surface. Dissociated cerebellar neurons were cultured on monolayers of control and transfected 3T3 cells and the sum of the length of neurites (a) or the length of the longest individual neurite (b) determined for 15–20 neurons from each of two independent cultures per clone for each experiment. The results show the mean $\pm$ s.e.m. value obtained for a total of at least 100 neurons from three or more independent experiments for cerebellar neurons cultured on control 3T3 monolayers ($\square$), or on monolayers expressing the secreted NCAM isoform ($\blacktriangle$), the 120K GPI-linked isoform (A8, G9, G18; $\circ$), the muscle-specific 125K GPI-linked isoform (G4, G9, G18; $\bullet$), or the transmembrane isoform (D1, D5; $\blacksquare$). The level of significance for the differences in means found between control and transfected 3T3 cells was determined by the single-sided Student's *t*-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

METHODS. Determination of the relative levels of NCAM expression. Approximately 2,000 cells from individual clones of transfected 3T3 cells were set up as eight replicate cultures in individual wells of a polylysine-coated 96-well microtitre plate. After ~ 18 h the cultures were fixed with paraformaldehyde and the binding of one of two independent monoclonal antibodies that recognize antigenic sites present on all human NCAM isoforms determined, as described previously¹². Antibody binding was normalized to the number of cells present in the wells at the end of the assay (as determined by direct cell counts), and the absolute level of antibody binding per cell then expressed in arbitrary units relative to the value obtained for clone D1 (100 units). During the assay, primary antibodies were present in vast excess and there was a linear relationship between antibody binding and cell number (tested for $0.5\text{--}5 \times 10^3$ cells). The specificity of antibody binding was demonstrated firstly by the failure to detect any significant binding to parental 3T3 cells plated at up to 20,000 cells per microwell, and secondly by the ability of PI-PLC to remove 95% of the binding signal from GPI-linked NCAM transfectants. The relative level of antibody binding between clones did not vary as a function of cell density (tested for $2\text{--}20 \times 10^3$ cells per microwell) and identical results were obtained with both monoclonal antibodies. The results show the mean relative binding determined for three to five independent experiments. The mean standard error on these values was 2.9 units (range 1–6). As well as the antibodies to human NCAM, 3T3 cells show no reactivity with monoclonal and polyclonal antibodies to mouse NCAM¹².

of neurite outgrowth is the relative level of NCAM expression, rather than the isoform type. This is perhaps not surprising, in view of the fact that all the above isoforms share a common NH_2 -terminal domain of ~ 500 amino acids which contains the site that mediates NCAM homophilic binding^{18,19}. An unexpected observation was the relatively sharp drop-off in neuronal responsiveness to NCAM; two clones that expressed about half of the level of NCAM as clone D1 were completely ineffective in enhancing neurite outgrowth. These data suggest that a discrete threshold value of NCAM is required for the expression of this function. Above this threshold value, small changes in NCAM expression can have substantial effects on neurite

FIG. 3 The effects of PI-PLC on NCAM expression at the a, cell surface of transfected cells and b, neurite outgrowth.

METHODS. Approximately 5,000 cells from a clone of transfected cells expressing GPI-linked NCAM (clone G9), or transmembrane NCAM (clone D1) were plated into the wells of a polylysine-coated 96-well plate. After ~ 18 h, PI-PLC (ICN Biomedicals, 0.05U per ml) was added and the cultures were maintained for a further 2, 4 or 26 h before being washed with DMEM and fixed and assayed for NCAM expression as described above. The results show the levels of NCAM expressed as percentages of that for cultures maintained in the absence of PI-PLC and each value represents the mean $\pm$ s.e.m. of six independent determinations. In b, co-cultures of cerebellar neurons on control and transfected 3T3 cells were established as described above that 0.05U per ml PI-PLC was added 2 h before the neurons. The level of neurite outgrowth was determined as in Fig. 2, and the results show this value after 21 h of co-culture. Each value is the mean $\pm$ s.e.m. determined for a total of at least 40 neurons from two duplicate cultures.



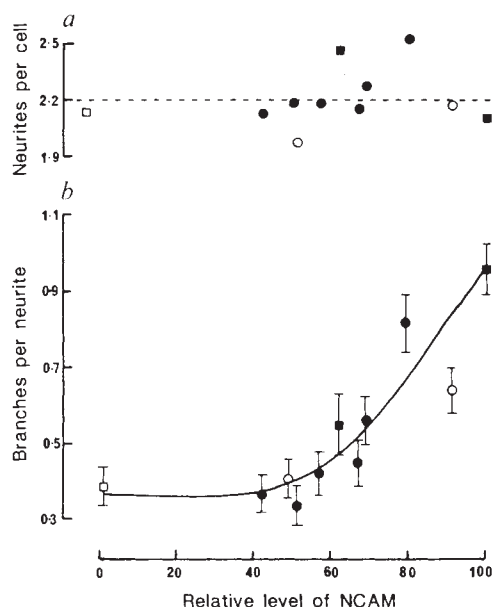


FIG. 4. Morphometric analysis of neurons cultured on control 3T3 monolayers ($\square$), monolayers expressing a secreted NCAM ($\blacktriangle$), monolayers expressing GPI-linked NCAM ($\odot$) or monolayers expressing transmembrane NCAM ($\blacksquare$). The total number of primary neurites per cell (a) and branch points per neurite (b) were determined for all neurons with one or more processes $>15\ \mu\text{m}$ (approximately 70–80% of the total population on both control and transfected monolayers). The results show these data as a function of the relative level of NCAM (see Fig. 2) and each value represents the mean of approximately 100 determinations. The average standard error in (a) was 0.082 (range 0.035–0.135) neurites per cell. None of the data points differed significantly from the control value.

outgrowth. For example, the expression of up to 70 arbitrary units of NCAM increases average neurite length by $\sim 45\ \mu\text{m}$, whereas the expression of 100 units increases average length by $175\ \mu\text{m}$ (Fig. 2a). Thus, a 40% increase in NCAM expression results in a 250% increase in the magnitude of the response. This highly cooperative relationship suggests that growth cones must establish contacts involving several, if not many, NCAM molecules at once before neurite outgrowth is affected.

To confirm that cell-associated NCAM induced neurite outgrowth directly, we established a series of co-cultures in media supplemented with phosphatidylinositol-specific phospholipase C (PI-PLC) at 0.05 U per ml. This enzyme completely removed GPI-linked NCAM from monolayers without affecting the level of expression of the transmembrane isoform (Fig. 3a). Cerebellar neurons express the 140K and 180K isoforms of NCAM, and PI-PLC has no significant effect on the level of NCAM immunoreactivity in cultures of these cells (data not shown). Although the enzyme had no effect on neurite outgrowth over monolayers of parental 3T3 cells, it completely abolished the differential growth normally associated with cells expressing the GPI-linked NCAM isoforms. In contrast, cells expressing the transmembrane isoform continued to support enhanced neuronal differentiation (Fig. 3b).

In addition to neurite length, we examined morphological parameters. The expression of NCAM did not affect the percentage of neurite-bearing cells, and had no effect on the number of primary neurites per cell (Fig. 4a). However, there were highly significant increases in the number of branches per neurite for neurons grown on monolayers expressing the higher levels of NCAM (Fig. 4b). There were no obvious differences in the branching pattern between neurons grown on monolayers expressing the different membrane-associated NCAM isoforms.

We have shown previously that the relative level of immunoreactive neurofilament protein is significantly increased when sensory neurons are grown on monolayers of transfected cells (3T3 and L cells) expressing the membrane-associated NCAM isoforms¹². Therefore, neurons of both the central and peripheral nervous systems must be able to recognize and respond to several distinct isoforms of NCAM by increased neurite outgrowth. The isoforms tested so far constitute all of the major classes of NCAM expressed by non-neuronal cells¹³. The demonstration that astrocytes and Schwann cells are preferential substrata for neurite outgrowth^{20,21} is likely to be at least partially due to their expression of NCAM, a view that is supported by recent antibody perturbation experiments^{8,9}.

In both peripheral nerve and muscle, individual NCAM isoforms undergo spatio-temporal changes in their pattern of expression following nerve transection that suggest that they have a physiological role in the promotion of neurite outgrowth^{22–25}. The work described here shows that a critical threshold level of NCAM is required before cerebellar neurons will respond to it. Above the threshold value, small changes in NCAM levels can have major effects on neurite outgrowth. We would postulate that the threshold value would be dependant on the level of NCAM both at the growth cone and on the cellular substratum, and on other factors, such as the polysialic acid content of this glycoprotein^{11,18}. This threshold hypothesis may account for the results of recent antibody perturbation experiments that suggest that retinal neurons from embryonic day 11 but not day 7 chicks are responsive to NCAM on astrocytes⁸, and similar experiments that show that ciliary ganglion neurons are responsive to NCAM on muscle⁶ but not NCAM on Schwann cells²⁶ or astrocytes⁷. $\square$

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- Dodd, J. & Jessell, T. M. *Science* **242**, 692–699 (1988).
- Edelman, G. M. *A. Rev. cell. Biol.* **2**, 81–116 (1986).
- Bastiani, H. J., Doe, C. Q., Helfand, S. L. & Goodman, C. S. *Trends Neurosci.* **8**, 257–266 (1985).
- Lagenaur, C. & Lemmon, V. *Proc. natn. Acad. Sci. U.S.A.* **84**, 7753–7757 (1987).
- Matsunaga, M., Hatta, K., Nagafuchi, A. & Takeichi, M. *Nature* **334**, 62–64 (1988).
- Bixby, J. L., Pratt, R. S., Lilien, J. & Reichardt, L. F. *Proc. natn. Acad. Sci. U.S.A.* **84**, 2555–2559 (1987).
- Tomaselli, K. J., Neugebauer, K. N., Bixby, J. L. & Reichardt, L. F. *Neuron* **1**, 33–43 (1988).
- Neugebauer, K. N., Tomaselli, K. J., Lilien, J. & Reichardt, L. F. *J. Cell Biol.* **107**, 1177–1187 (1988).
- Seilheimer, B. & Schachner, M. *J. Cell Biol.* **107**, 341–351 (1988).
- Chang, S., Rathjen, F. G. & Raper, J. A. *J. Cell Biol.* **104**, 355–362 (1987).
- Rutishauser, U., Acheson, A., Hall, A. K., Mann, D. M. & Sunshine, J. *Science* **240**, 53–57 (1988).
- Doherty, P. *et al. J. Cell Biol.* **109**, 789–798 (1988).
- Nybroe, O., Linnemann, D. & Bock, E. *Neurochem. Int.* **12**, 251–262 (1988).
- Gower, H. J. *et al. Cell* **55**, 955–964 (1988).
- Walsh, F. S. *et al. Development* **105**, 803–811 (1989).
- Dickson, G. *et al. Cell* **50**, 1119–1130 (1987).
- Thompson, J. *et al. Genes Dev.* **3**, 348–357 (1989).
- Frellinger, A. L. & Rutishauser, U. *J. Cell Biol.* **103**, 1729–1737 (1986).
- Cunningham, B. A. *et al. Science* **236**, 799–806 (1987).
- Noble, M., Fok-Seang, J. & Cohen, J. *J. Neurosci.* **4**, 1892–1903 (1984).
- Fallon, J. R. *J. Neurosci.* **12**, 3169–3177 (1985).
- Daniloff, J. K., Levi, G., Grumet, M., Rieger, F. & Edelman, G. M. *J. Cell Biol.* **103**, 929–945 (1986).
- Covault, J. & Sanes, J. R. *Proc. natn. Acad. Sci. U.S.A.* **82**, 4544–4548 (1985).
- Moore, S. E. & Walsh, F. S. *Neuroscience* **18**, 499–505 (1986).
- Martini, R. & Schachner, M. *J. Cell Biol.* **106**, 1735–1746 (1987).
- Bixby, J. L., Lilien, J. & Reichardt, L. F. *J. Cell Biol.* **107**, 353–361 (1988).
- Finne, J., Finne, U., Deagostini-Bazin, H. & Goridis, C. *Biochem. biophys. Res. Commun.* **112**, 482–487 (1983).
- Rougon, G., Dubois, C., Buckley, N., Magnani, J. L. & Zollinger, W. *J. Cell Biol.* **103**, 2429–2437 (1986).

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Binding of a *Drosophila* POU-domain protein to a sequence element regulating gene expression in specific dopaminergic neurons

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SEVERAL genes have been identified that affect the specification of unique neuronal identities during development of the *Drosophila* central nervous system¹⁻⁷. At least two of these genes, *fushi tarazu* (*ftz*)⁴ and *even-skipped* (*eve*)⁵ share a common structural motif, the homoeobox, which encodes a sequence-specific DNA-binding domain (homoeodomain)⁸. A family of related proteins has been recently characterized in mammals and nematodes that contain a diverged homoeodomain as part of a structure referred to as a POU domain⁹⁻¹⁵. Mammalian genes encoding POU domains have region-specific patterns of expression in the central nervous system¹⁵, indicating a potential role for them in the regulation of neuronal development. The nematode POU-domain gene, *unc-86*, is involved in the determination of neuroblast lineages leading to serotonergic and dopaminergic neurons^{13,14}. We have now identified a *Drosophila* gene, *Cfla*, encoding a sequence-specific DNA-binding protein containing a highly conserved POU domain. The *Cfla* gene product binds to a DNA element required for expression

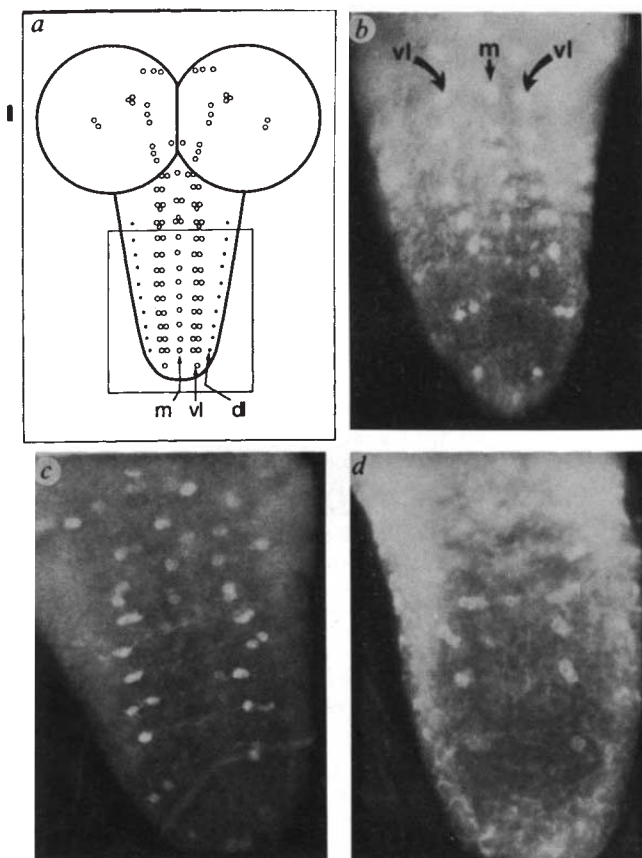
of the dopa decarboxylase gene (*Ddc*) in selected dopaminergic neurons, implying that it functions as a neuron-specific transcription factor.

The *Ddc* gene encodes the last enzyme in a pathway leading to synthesis of the neurotransmitters dopamine and serotonin. The precise pattern of neuronal *Ddc* expression (Fig. 1) requires at least two regulatory regions, a binding site for the factor *Elf1* (ref. 16) at -60 base pairs (bp) and a ~600 bp distal enhancer¹⁷. Specific regions of the *Ddc* distal enhancer are essential for *Ddc* expression in defined subsets of neurons¹⁷. These regions contain binding sites for several DNA-binding factors present in crude embryonic nuclear extracts (Fig. 2a). We have used oligonucleotide-directed *in vitro* mutagenesis to introduce a clustered point mutation disrupting one of these factor-binding sites. The Cfl1-binding site (Fig. 2a; ref. 17) is associated with an evolutionarily conserved sequence element, element C. An 8-bp clustered point mutation within element C abolished the binding of factor(s) to the Cfl1-binding site *in vitro* (Fig. 2a, b; lanes 1-4) and had no effect on the *in vitro* binding of other factors within the distal enhancer (Fig. 2b, and data not shown).

P-element transformed strains expressing a *Ddc* gene containing the altered element C (*Ddc*^{Cmut124}), showed an absence or severe reduction of *Ddc* immunoreactivity in the dopaminergic medial neurons of the ventral ganglion, whereas all other *Ddc*-containing neurons seemed to express *Ddc* at normal levels (Fig. 1c). So, the *Ddc*^{Cmut124} mutant phenotype not only distinguishes

FIG. 1 *In vivo* expression of wild-type *Ddc* and *Ddc*^{Cmut124} genes. a, Line drawing of a wild-type larval central nervous system showing neurons containing *Ddc* immunoreactivity after labelling by indirect immunofluorescence using an anti-*Ddc* antisera¹⁸. Wild-type strains of *D. melanogaster* normally express *Ddc* in ~150 neurons in the segmented ventral ganglion and the brain lobes^{18,24}. We concentrated our analysis on neurons within the ventral ganglion, where segmentally repeated units contain two symmetrical rows of doublet cells, the ventrolateral neurons (vl), a single row of midline cells, the medial neurons (m), and two additional symmetrical rows of single cells, the dorsolateral neurons (dl). The ventrolateral cells are serotonergic neurons¹⁸, whereas the medial and dorsolateral cells are dopaminergic neurons²⁵. b, Higher magnification fluorescence photomicrograph of a wild-type ventral ganglion. Area indicated by boxed region of a. Representative medial (m) and ventrolateral (vl) neurons are indicated by black arrows. c, A *Ddc*^{Cmut124} ventral ganglion shows severely diminished levels of *Ddc* immunoreactivity in dopaminergic medial neurons, but nearly wild-type levels in serotonergic ventrolateral neurons. Dopaminergic dorsolateral neurons are not visible in this focal plane but show wild-type levels of *Ddc* immunoreactivity. The phenotype displays a distinct anterior-posterior differential, with the most pronounced effect on the abdominal neuromeres corresponding to segments A2-A8. A moderate effect is seen in neuromeres T3 and A1, whereas more anterior dopaminergic neurons within the sub-oesophageal ganglia show virtually no effect. *Ddc*^{Cmut124} strains do not show the overexpression of *Ddc* in glial cells seen in strains expressing *Ddc* genes with the entire distal enhancer deleted¹⁸. d, A *Ddc*^{EN5Δ199} ventral ganglion from a transformant strain expressing a *Ddc* gene with 199 bp deleted from the 5' end of the minimal distal enhancer¹⁷, shows a nearly identical phenotype with selective loss of *Ddc* immunoreactivity in dopaminergic medial neurons. Note, however, the increased levels of *Ddc* immunoreactivity in glial cells, which normally express *Ddc* at very low levels^{17,18}.

METHODS. *Ddc* genes containing clustered point mutations were cloned into a P-element vector containing the *Drosophila* alcohol dehydrogenase gene (*Adh*) as a selectable marker. Nucleic acid manipulations were mainly as previously described²⁶ except where indicated. Restriction enzymes were purchased from New England Biolabs or Boehringer Mannheim Biochemicals and used as suggested by the supplier. Methods for P-element integration were essentially as described previously^{27,28}. The host strain was *Adh^{fn23}Ddc^{ts2}*. Transformant flies were identified by screening for wild-type *Ddc* pupal phenotype at 25 °C. Five independent transformant strains were established for each *Ddc* mutation. Strains were confirmed to contain single copy inserts of the appropriate P-element vector by Southern-blot analysis of genomic DNA. Anti-*Ddc* antiserum and protocols for immunofluorescent detection of *Ddc* in the isolated central nervous system of third instar larvae were as previously described¹⁸.



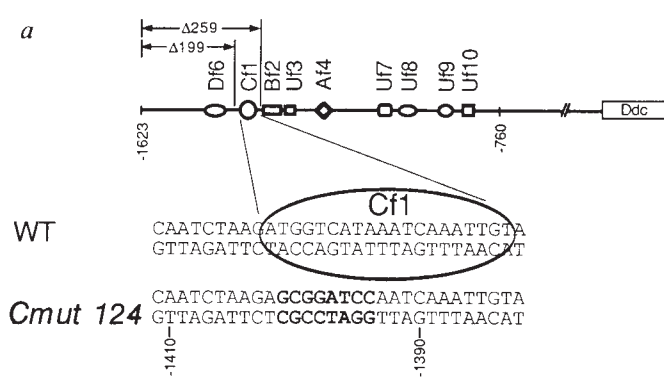
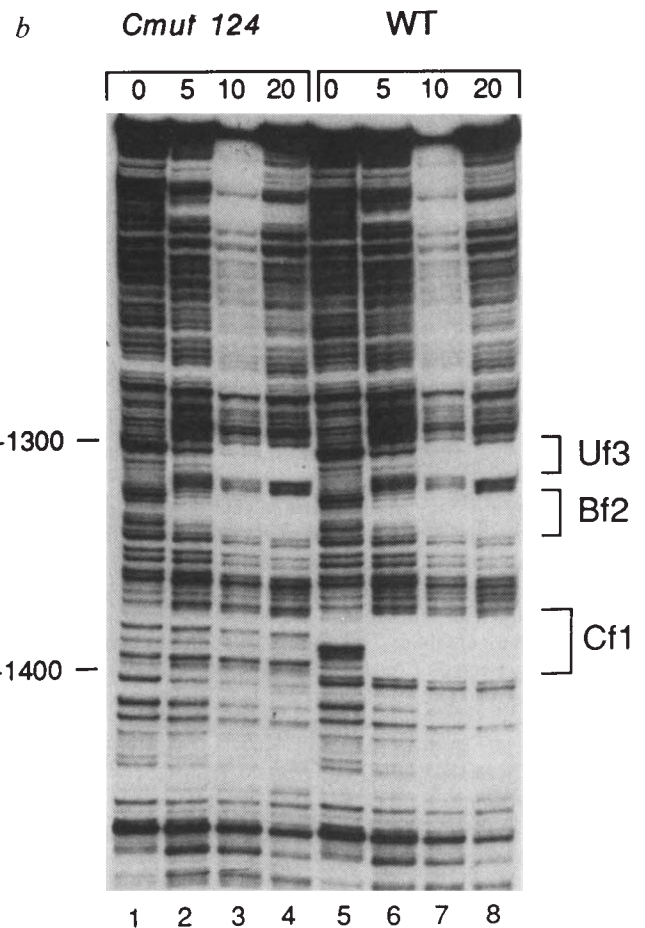


FIG. 2 DNase I protection of wild-type (WT) and mutant element C. **a**, Schematic representation of identified factor binding sites in the *Ddc* distal enhancer¹⁷ with deletion endpoints indicated for *Ddc*^{EN5'Δ199} and *Ddc*^{EN5'Δ259}. Expanded nucleotide sequence shows the Cf1-binding site, which extends from -1,382 to -1,402 bp relative to the mRNA start site in the wild-type conserved sequence element, element C. *Ddc*^{Cmut124} contains an 8-bp clustered point mutation extending from -1,394 to -1,401 bp, as shown in bold. **b**, DNase protection of wild-type and mutant Cf1-binding site by crude embryonic nuclear extracts. Single-end ³²P-labelled fragments from wild-type (lanes 5–8) and *Ddc*^{Cmut124} (lanes 1–4) genes were incubated with increasing quantities of embryonic nuclear extract as indicated at top of each lane (μl per 50 μl reaction) before digestion with DNase I. Regions of protection by factors Cf1, Bf2 and Uf3 are indicated by brackets on the right. Factor Cf1 no longer binds to the *Cmut124* binding site (lanes 1–4), whereas the binding of factors Bf2 and Uf3 is unchanged. The footprint representing binding of factor Df6 is not visible within the range of this gel. **METHODS.** Clustered point mutations were generated by oligonucleotide-directed *in vitro* mutagenesis²⁹. DNA sequences were determined by dideoxy-chain-termination sequencing using modified T7 DNA polymerase³⁰. Nuclear extracts were prepared from 10–22-h embryos and fractionated on heparin agarose³¹. Footprinting reactions were performed as previously described³², using proteins eluted from heparin agarose between 0.1 and 0.4 M KCl. One microlitre of synthetic poly(dI-dC) (Pharmacia; 10 U ml⁻¹) was added to each reaction as nonspecific competitor. Radioactive probes were prepared by labelling the 860-bp *Xba*I–*Hind*III fragments (-1,623 bp to



-760 bp) from wild-type *Ddc*^{Cmut124} genes at the unique *Xba*I site using polynucleotide kinase. Digested fragments were analysed on 7% polyacrylamide sequencing gels.

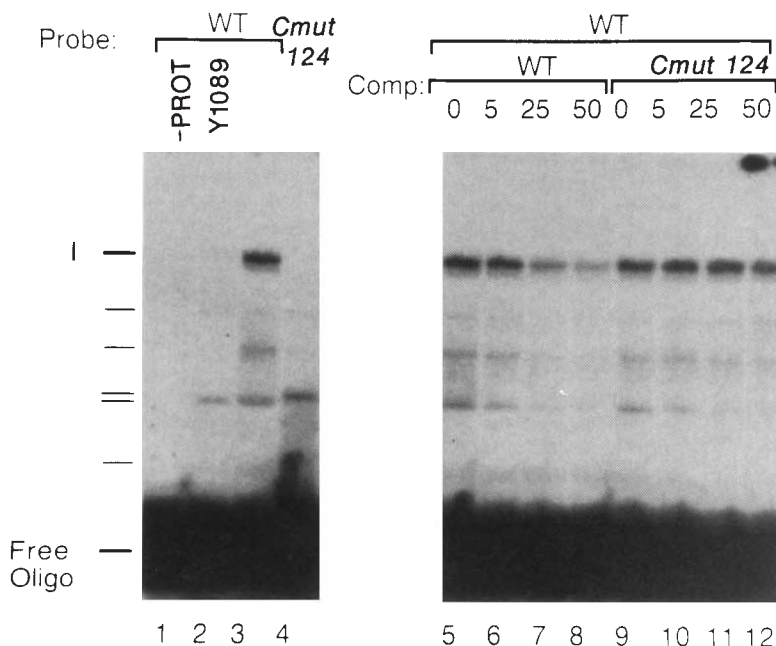


FIG. 3 *In vitro* binding of the β -galactosidase-Cf1a fusion protein. **a**, Electrophoretic mobility shift assay showing the presence of IPTG-induction-dependent binding to the wild-type Cf1-binding site. Lanes 1–3, ³²P-labelled Cf1-binding-site oligonucleotide; lane 4, ³²P-labelled *Cmut124* oligonucleotide. Lane 1, no-protein control. Lane 2, extract prepared from nonlysogenized IPTG-induced Y1089. Lanes 3–4, extract prepared from IPTG-induced λ Cf1a lysogen. The most intense and slowest-migrating complex is labelled I; other nonspecific complexes faintly present in all lanes 2–12 are indicated by the lines to the left of the gel. **b**, Inhibition of complex formation by excess unlabelled oligonucleotides. Reaction mixtures for all lanes contained ³²P-labelled double-stranded Cf1-binding site oligonucleotide with extract prepared from IPTG-induced λ Cf1a lysogen. Increasing concentrations of unlabelled double-stranded wild-type Cf1-binding-site and mutant *Cmut124* oligonucleotides were added as indicated (ng per 8 μl reaction).

METHODS. Whole-cell lysates were prepared from non-lysogenized Y1089 cultures or from IPTG-induced λ Cf1a lysogen²¹. Gel-shift binding assays were performed as previously described³³. Reaction mixtures (8 μl) contained 1 μl 10% polyvinyl alcohol and 0.2 U poly(dI-dC)-poly(dI-dC) as nonspecific competitor with indicated concentration of unlabelled double-stranded oligonucleotides. Four microlitres of appropriate extracts containing 8–10 μg total protein were added to each reaction. After incubation at 4 °C for 15 min, reactions were electrophoresed through 5% native polyacrylamide gels in 1 × Tris-borate buffer. WT, wild type; Oligo, oligonucleotide.

between dopaminergic and serotonergic neurons, but also defines anatomical subsets of dopaminergic neurons that regulate *Ddc* expression by different mechanisms. Deletion of the entire distal enhancer has no effect on *Ddc* expression in the hypoderm⁸, where the *Ddc* enzyme is an essential component in cuticle formation^{19,20}. *Ddc*^{Cmut124} strains display wild-type *Ddc* pupal phenotypes, and levels of *Ddc* enzyme activity in the hypoderm are normal (data not shown). A nearly identical phenotype results from two progressive deletions of the *Ddc* distal enhancer, *Ddc*^{EN5'Δ199} and *Ddc*^{EN5'Δ259} (Figs 1d and 2a, and ref. 17). Because the Δ199 deletion does not remove the Cfl-binding site, there must be at least one unidentified essential dopaminergic-specific regulatory element in addition to element C.

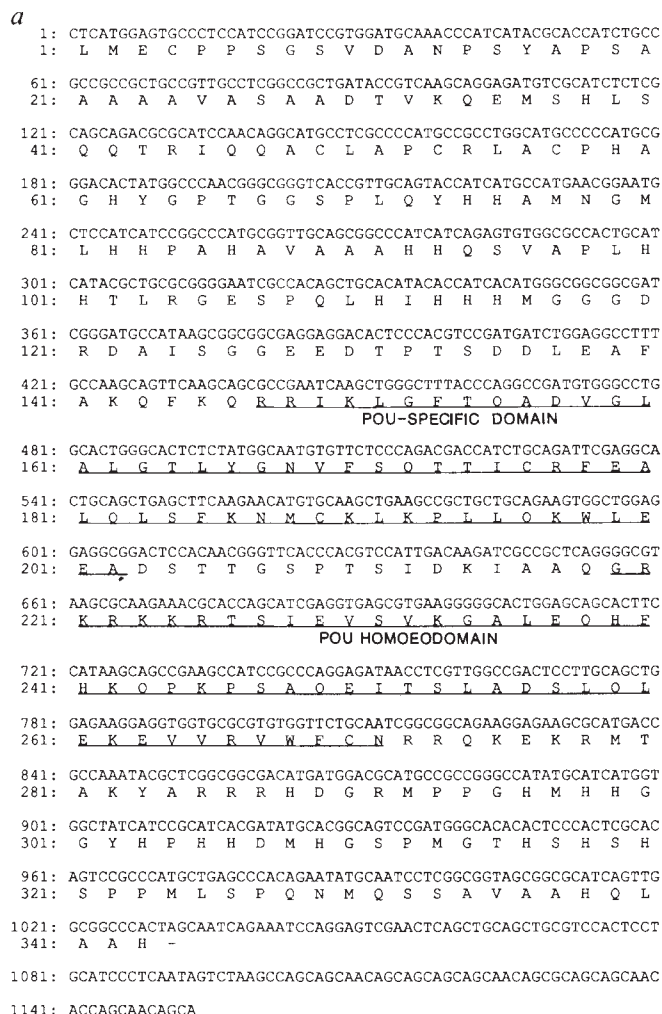
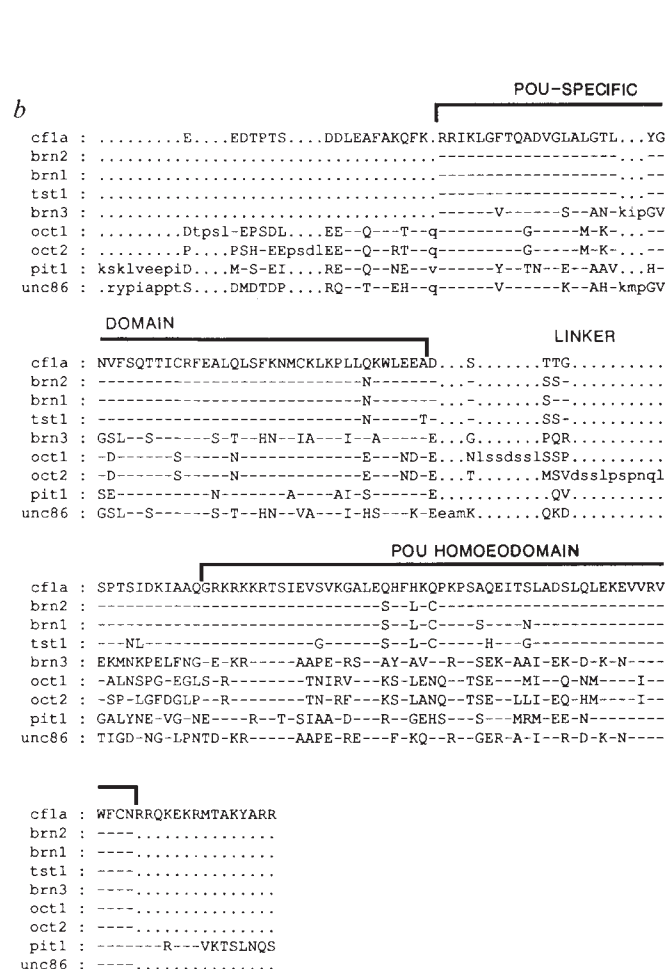


FIG. 4 Conservation of the POU domain in the predicted *Cfla* translation product. **a**, Nucleotide sequence and predicted translation product (single-letter amino-acid code) of the λ Cfl1a 1029-bp open reading frame encoding 343 amino acids with its N terminus corresponding to the site of β -galactosidase fusion. Regions corresponding to the POU-specific and POU homoeodomains are underlined. **b**, Amino-acid sequence comparison of the *Cfla* POU domain with previously isolated POU domains. Amino-acid sequences for Pit-1, Oct-1, Oct-2 and Unc-86 are as determined by Herr *et al.*³⁴ and the sequences Brn-1, Brn-2, Tst-1 and Brn-3 sequences by He *et al.*¹⁵. The POU homoeodomain and POU-specific domains are indicated by brackets. Dashes indicate amino-acid identities and dots correspond to gaps in the sequence. The sequence of the λ Cfl1a insert has been submitted to GenBank. METHODS. The positive λ Cfl1a phage was isolated by screening an embryonic λ gt11 expression library (a gift of Tao Hsieh, Duke University Medical School) with a ³²P-labelled concatenated oligonucleotide containing the wild-type *Cfl1*-binding site using a modification of protocols described by Vinson *et al.*²². After the guanidine denaturation-renaturation cycle, 0.1%

In an attempt to identify the factor or factors binding *in vivo* to element C, we have screened an embryonic λ gt11 expression library^{21,22} to isolate a phage, λ Cfl1a, whose expression product specifically binds a ³²P-labelled concatenated oligonucleotide with Cfl1-binding sites. The relationship between Cfl1-specific DNA-binding activity and the β -galactosidase fusion protein expressed by λ Cfl1a was demonstrated by examining the binding specificity of the protein in *Escherichia coli* extracts. Whole cell extracts prepared from isopropyl- β -D-thiogalactopyranoside (IPTG)-induced λ Cfl1a lysogen as well as non-lysogenized Y1089 cells were examined by SDS-PAGE (data not shown). A high relative molecular mass protein ($M_r \sim 150,000$) was present in extracts from IPTG-induced lysogen, but was not detected in uninduced or nonlysogenized extracts. Several slowly migrat-



Nonidet P-40 was added to all wash buffers and to the binding reaction. The binding reaction (50 μ l) also contained 5 U poly (di-dC)-(di-dC). Positive plaques obtained from the initial screen of $\sim 5 \times 10^5$ clones were rescreened for differential binding of the Cfl1-binding-site oligonucleotide and a control nonspecific oligonucleotide. The λ Cfl1a insert was subcloned into Bluescript SK vector (Stratagene) for restriction mapping and sequencing. The complete 3-kb λ Cfl1a insert is a double insertion clone with an internal *EcoRI* site associated with a long poly(A) stretch ~ 2 kb from the N terminus. Fusion with the λ gt11 β -galactosidase gene is at the N terminus of the open reading frame encoding the Cfl1a POU domain. Many termination signals in all reading frames between the POU-domain-encoding sequences and the internal *EcoRI* site indicate that this was the only reading frame translated in the expression screen. DNA sequences were determined by dideoxy nucleotide sequencing techniques, using modified T7 DNA polymerase³⁰. Amino-acid alignment was performed using the program of Altschul and Erickson³⁵ as implemented in EUGENE (Baylor College of Medicine).

ing complexes resulted from gel-shift assays of reactions containing ^{32}P -labelled Cfl1-binding-site oligonucleotide and extracts of the induced λCfl1a lysogen (Fig. 3a, lane 3). Only complex I, the slowest-migrating form, results from a specific interaction with the Cfl1-binding site, because it does not form when induced λCfl1a extracts are incubated with a ^{32}P -labelled *Cmut124* oligonucleotide (Fig. 3a, lane 4). Extracts from induced non-lysogenized Y1089 cells (Fig. 3, lane 2), as well as noninduced λCfl1a lysogen (data not shown), failed to form the specific complex I. Formation of complex I was competitively inhibited by unlabelled Cfl1-binding-site oligonucleotide (Fig. 3b, lanes 5–8), but was unaffected by the addition of unlabelled *Cmut124* oligonucleotide (Fig. 3b, lanes 9–12).

The λCfl1a insert was analysed by DNA sequencing and predicted to encode a protein containing a highly conserved POU domain of 143 amino acids in the C-terminal portion (Fig. 4a). Alignment of the entire Cfl1a POU domain with predicted amino-acid sequences from previously identified mammalian and nematode POU domains shows a remarkable interspecies sequence conservation (Fig. 4b). The human complementary DNAs *Brn-1* and *Brn-2* potentially encode proteins with the most significant conservation, retaining >95% amino-acid identity in the 143-amino-acid Cfl1a POU domain. In addition, the normally diverged linker of 17 amino acids between the homoeodomain and POU-specific domain is highly conserved between Cfl1a and the proteins encoded by *Brn-1*, *Brn-2* and *Tst-1* cDNAs. If the terminology of He *et al.*¹⁵ is used, Cfl1a would belong to the POU-III class of POU proteins.

We have localized the *Cfl1a* gene to region 65D of the third chromosome (data not shown) immediately adjacent (<100 kb) to the locus encoding tyrosine hydroxylase (TH) at 65C. TH is presumed to be the rate-limiting enzyme in the synthetic pathway responsible for catecholamine biosynthesis²³. The coincidental location of the *Cfl1a* gene near a gene expressed in dopaminergic neurons allows speculation about the existence of a dopaminergically expressed gene cluster encompassing both *TH* and *Cfl1a*.

Cfl1a is structurally related to members of a group of previously characterized neurally expressed genes^{9–15}, one of which, the nematode gene *unc-86*, functions in the specification of dopaminergic and serotonergic neurons^{13,14}. The data described here support the suggestion that the POU-domain-containing Cfl1a directly regulates *Ddc* expression in selected dopaminergic neurons through the Cfl1-binding site. But specific *in vitro* recognition of this binding site does not necessarily imply an *in vivo* function as there may be multiple proteins capable of *in vitro* and/or *in vivo* recognition of a single binding site.

It is likely that the *Cfl1a* gene represents a single member of a group of *Drosophila* POU-domain proteins yet to be identified. At least two *Drosophila* POU-domain genes, one of which is presumed to be identical to *Cfl1a*, have been isolated using the polymerase chain reaction (M. Treacy, personal communication). The region-specific expression of many POU-domain proteins in the central nervous system¹⁵ indicates that conserved characteristics of structure and tissue distribution extend across several evolutionary barriers. This implies a fundamental requirement for these genes during neuronal development. The isolation of a *Drosophila* POU-domain gene opens up many possibilities for genetic characterization of function, and for studies of *in vivo* gene regulation, during development. □

11. Sturm, R. A., Das, G. & Herr, W. *Genes Dev.* **2**, 1582–1599 (1988).
12. Clerc, R. G., Corcoran, L. M., LeBowitz, J. H., Baltimore, D. & Sharp, P. A. *Genes Dev.* **2**, 1570–1581 (1988).
13. Finney, M., Ruvkun, G. & Horvitz, H. R. *Cell* **55**, 757–769 (1988).
14. Desai, C., Garriga, G., McIntire, S. L. & Horvitz, H. R. *Nature* **336**, 638–646 (1988).
15. He, X. *et al.* *Nature* **340**, 35–42 (1989).
16. Bray, S. J., Johnson, W. A., Hirsh, J., Heberlein, U. & Tjian, R. *EMBO J.* **7**, 177–188 (1988).
17. Johnson, W. A., McCormick, C. A., Bray, S. J. & Hirsh, J. *Genes Dev.* **3**, 676–686 (1989).
18. Beall, C. & Hirsh, J. *Genes Dev.* **1**, 510–520 (1987).
19. Luman, K. D. & Mitchell, H. K. *Archs Biochem. Biophys.* **132**, 450–456 (1969).
20. Wright, T. R. F., Bewley, G. C. & Sherald, A. F. *Genetics* **84**, 287–310 (1976).
21. Singh, H., LeBowitz, J. H., Baldwin, A. S., Jr & Sharp, P. A. *Cell* **52**, 415–423 (1988).
22. Vinson, C. R., LaMaraco, K. L., Johnson, P. F., Landschutz, W. H. & McKnight, S. L. *Genes Dev.* **2**, 801–806 (1988).
23. Neckameyer, W. S. & Quinn, W. G. *Neuron* **2**, 1167–1175 (1989).
24. Konrad, K. D. & Marsh, J. L. *Devl Biol.* **122**, 172–185 (1987).
25. Budnick, V. & White, K. J. *comp. Neurol.* **268**, 400–413 (1988).
26. Maniatis, T., Fritsch, E. & Sambrook, J. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, New York, 1982).
27. Rubin, G. M. & Spradling, A. C. *Science* **218**, 348–353 (1982).
28. Scholnick, S., Morgan, B. A. & Hirsh, J. *Cell* **34**, 37–45 (1983).
29. Taylor, J. W., Ott, J. & Eckstein, F. *Nucleic Acids Res.* **13**, 8765–8785 (1985).
30. Tabor, S. & Richardson, C. C. *Proc. natn. Acad. Sci. U.S.A.* **84**, 4767–4771 (1987).
31. Heberlein, U. & Tjian, R. *Nature* **331**, 410–415 (1988).
32. Heberlein, U., England, B. & Tjian, R. *Cell* **41**, 965–977 (1985).
33. Bray, S. J., Burke, B., Brown, N. H. & Hirsh, J. *Genes Dev.* **3**, 1130–1145 (1989).
34. Herr, W. *et al.* *Genes Dev.* **2**, 1513–1516 (1988).
35. Altschul, S. F. & Erickson, B. W. *Bull. math. Biol.* **48**, 603–616 (1986).

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Potent and selective inhibition of HIV-1 replication *in vitro* by a novel series of TIBO derivatives

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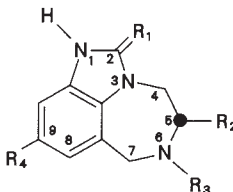
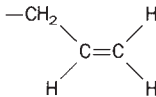
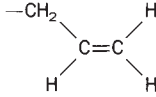
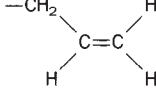
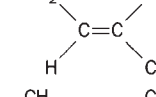
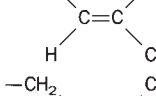
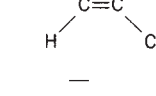
IN the search for compounds active against human immunodeficiency virus (HIV), we have found that members of a novel series of tetrahydro-imidazo[4,5,1-jk][1,4]-benzodiazepin-2(1H)-one and -thione (TIBO) derivatives inhibit the replication of HIV-1 (refs 1, 2), the main aetiological agent of AIDS, but not of HIV-2 (ref. 3), or of any other DNA or RNA viruses. In five cell systems, HIV-1 is inhibited by TIBO derivatives in nanomolar amounts, which are 10^4 – 10^5 times lower than the cytotoxic concentration. The unprecedented specificity of these compounds may be due to an interaction with a reverse transcriptase-associated process. By contrast, AZT (3'-azido-2',3'-dideoxythymidine), which is used for the treatment of AIDS, and DDC (2',3'-dideoxycytidine) and DDI (2',3'-dideoxyinosine), whose clinical application is being assessed, inhibit both HIV-1 and HIV-2 at concentrations that, depending on the cell systems, are 2 to 4 orders of magnitude below their cytotoxic concentration^{4–8}. TIBO-derivatives are new chemicals unrelated to any other antiviral agents. We believe that they are the most specific and potent inhibitors of HIV-1 replication studied so far.

Our strategy to find new anti-HIV compounds with acceptable pharmacology started with the selection of 600 molecules, all prototypes of different chemical series, which were without effect in standard pharmacological assays⁹ and caused only mild toxicity in rodents. They were assayed for inhibition of multiple cycle growth and cytopathicity of HIV-1, and for cytotoxicity, in a

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1. Campos-Ortega, J. A. *Trends Neurosci.* **11**, 400–405 (1988).
2. Rubin, G. M. *Cell* **57**, 519–520 (1989).
3. Doe, C. Q. & Goodman, C. S. *Cold Spring Harb. Symp. quant. Biol.* **50**, 891–903 (1985).
4. Doe, C. Q., Hiromi, Y., Gehring, W. J. & Goodman, C. S. *Science* **239**, 170–175 (1988).
5. Doe, C. W., Smouse, D. & Goodman, C. S. *Nature* **333**, 376–378 (1988).
6. Patel, N. H., Schafer, B., Goodman, C. S. & Holmgren, R. *Genes Dev.* **3**, 890–904 (1989).
7. Thomas, J. B., Crews, S. T. & Goodman, C. S. *Cell* **52**, 133–141 (1988).
8. Gehring, W. J. & Hiromi, Y. *A. Rev. Genet.* **20**, 147–173 (1986).
9. Ingraham, H. A. *et al.* *Cell* **55**, 519–529 (1988).
10. Bodner, M. *et al.* *Cell* **55**, 505–518 (1988).

TABLE 1 Structure-activity relationship for inhibition of HIV-1 cytopathicity in MT-4 cells by tetrahydro-imidazo[4,5,1-jk][1,4]-benzodiazepin-2-(1H)-one and -thione (TIBO) derivatives

Compound	Molecular weight					IC ₅₀ (μ M)	CC ₅₀ (μ M)	Selectivity index
		R ₁	R ₂	R ₃	R ₄			
R14458	243.3	O	($\pm$) CH ₃		H	62	612	10
R78304	243.3	O	(-)-(R)CH ₃		H	> 500	592	<1
R78305	243.3	O	(+)-(S)CH ₃		H	70	674	10
R79882	271.4	O	($\pm$) CH ₃		H	13	840	65
R82150	287.4	S	(+)-(S)CH ₃		H	0.028	>870	>31,071
R82913	321.9	S	(+)-(S)CH ₃		Cl	0.0015	31	20,667
AZT	267.2	—	—	—	—	0.0015	9.3	6,200
DDC	211.2	—	—	—	—	0.137	232	1,693
DDI	236.2	—	—	—	—	5.5	1,053	191

Data are median values of at least 10 experiments. IC₅₀ is the 50% inhibitory concentration for cytopathicity by HIV-1/HTLV-III_B (from R. C. Gallo) in MT-4 cells (from N. Yamamoto). The viral dose was 100–500-fold higher than necessary to infect 50% of the MT-4 cells. CC₅₀ is the 50% cytotoxic dose in mock-infected MT-4. The CC₅₀/IC₅₀ ratio is the selectivity index (SI). Compounds were first dissolved in dimethylsulphoxide. Viability was quantified by a tetrazolium colourimetric method on 96-well microtrays⁷.

sensitive MT-4 cell system^{7,10}, effectively being screened for their effects on combined target sites, rather than on isolated proteins. After the discovery of moderate but specific anti-HIV-1 activity in the prototype compound R14458 (Table 1), we synthesized and evaluated many congeners to elucidate the relationship between their structure and activity and to enhance their potency stepwise.

As R14458 is a racemic mixture, we first synthesized its two optical isomers, R78304 and R78305, with configurations (-)-(R) and (+)-(S) respectively. Only the (+)-(S) isomer inhibits HIV-1. This requirement for the (+)-(S) configuration for anti-HIV-1 activity is common to all the TIBO derivatives we synthesized (data not shown). We found that substitution of the allyl side chain by 3-methyl-2-butenyl (R79882) gave a 6-fold increase in potency, and replacement of oxygen by sulphur at the C2 position of the imidazole ring resulted in a further 450-fold increase in potency. In this R82150 prototype TIBO-derivative, the anti-HIV-1 activity of compound R14458 is enhanced by more than 2,000-fold, without increasing toxicity to the host cells. By introducing a chlorine at the C-9 position of the phenyl moiety (R82913), the high potency of AZT in the MT-4 system is equalled and its selectivity surpassed by 3-fold (Table 1). Compound R82150, (+)-S-4,5,6,7-tetrahydro-5-methyl-6-(3-methyl-2-butenyl)-imidazo[4,5,1-jk][1,4]-benzodiazepin-2(1H)-thione (melting point 174.5 °C) was synthesized in an eleven-step

procedure starting from 2-methyl-6-nitroaniline, with an overall yield of 3–4%. The asymmetric centre was introduced by incorporation of (S)-alanine. The solubility was 0.14 mg ml⁻¹ in water and 4.21 mg ml⁻¹ in a citrate-phosphate buffer, pH 4. Compound R82913, (+)-S-4,5,6,7-tetrahydro-9-chloro-5-methyl-6-(3-methyl-2-butenyl)-imidazo[4,5,1-jk][1,4]-benzodiazepin-2(1H)-thione (melting point 178–181 °C), was synthesized by an eight-step procedure starting from 5-chloroisatoic anhydride, with an overall yield of 1–2%. The solubility was 0.002 mg ml⁻¹ in water and 0.54 mg ml⁻¹ in a citrate-phosphate buffer, pH 4. Although these new tetrahydro-benzodiazepine derivatives share some structural features with the classical dihydro-benzodiazepines, they contain a unique tricyclic system, and they are devoid of diazepam-like effects in rodents. We found no anti-HIV-1 activity in a large variety of classical benzodiazepines (data not shown).

Table 2 shows how the activity of R82150 extends to cells other than the HTLV-I-transformed MT-4 cells, and to viruses other than the prototype HTLV-III_B strain of HIV-1. In HTLV-I-free CEM and MOLT-4 lymphocyte lines, inhibitory concentrations against HTLV-III_B were similar to the ~28 nM value found in MT-4 cells. The selectivity index is of the same order in all cells. In fresh peripheral blood lymphocyte cultures, R82150 inhibits p24 protein production by 50% at 143 nM (Table 2) and completely at 0.7 μ M; the cytotoxic concentration is 800 μ M.

TABLE 2 Antiviral activity spectrum of the TIBO-derivative R82150, DDC and AZT

Virus	Strain	Cell	Assay	Day of analysis	R82150			DDC			AZT		
					IC ₅₀ (μM)	CC ₅₀ (μM)	SI	IC ₅₀ (μM)	CC ₅₀ (μM)	SI	IC ₅₀ (μM)	CC ₅₀ (μM)	SI
HIV-1	III _B	MT-4*	CPE/MTT	5	0.028	>870	>31,071	0.137	232	1,693	0.0015	9.3	6,200
		CEM†	CPE/MTT	8-10	0.017	421	24,765	0.019	1.8	95	0.0045	97	21,556
		MOLT-4†	CPE	8-10	0.010	—	—	0.014	—	—	0.0060	—	—
			MTT	8-10	—	518	51,800	—	1.4	100	—	105	17,500
		PBL‡	p24 antigen	12	0.143	—	—	0.08	—	—	0.0374	—	—
	III _{BA-L}	M/M§	p24 antigen	12	—	800	5,594	—	11	137	—	52	1,390
			MTT	12	—	—	—	—	—	—	—	—	—
		RF*	p24 antigen	14	0.010	ND	—	ND	ND	ND	0.0075	ND	—
		MT-4	CPE/MTT	5	0.010	>870	>87,000	0.147	232	1,578	0.0011	9.3	8,454
		HE*	CPE/MTT	5	0.100	>870	>8,700	0.019	232	12,210	0.0004	9.3	23,250
HIV-2	ROD*	MT-4	CPE/MTT	5	>870	>870	≥1	0.100	232	2,320	0.0011	9.3	8,454
	EHO*	MT-4	CPE/MTT	5	>870	>870	≥1	0.118	232	1,966	0.0008	9.3	11,625
	SIV	MAC-251*	CPE/TB	5	>150	>150	≥1	ND	ND	ND	ND	ND	ND
MSV	Moloney	C3H	Transformation	5	>150	>150	≥1	ND	ND	ND	ND	ND	ND

* The inhibition of HIV-1 (HTLV-III_B, RF²¹, HE), HIV-2 (ROD²² and EHO²³, provided by L. Montagnier) and SIV (MAC-251²⁴, provided by C. Bruck) was assayed in MT-4 cells as described in the legend to Table 1. Viability in anti-SIV assay was determined by trypan blue dye exclusion¹⁰.

† CEM and MOLT-4 (Clone number 8) are CD4⁺ leukaemic T-cell lines; antiviral assays were as described for MT-4 cells, but a virus preparation concentrated 100-fold was used. Cells were subcultured every 4 days without addition of new compound. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) colorimetric method⁷ at 8 to 10 days post-infection (p.i.) (CPE/MTT) (CEM) or by microscopic evaluation of syncytial cytopathic effect (CPE) (MOLT-4).

‡ Peripheral blood lymphocytes (PBL) were obtained by the Ficoll-Hypaque technique and stimulated with phytohaemagglutinin (Wellcome) for 3 days at 37 °C. These cells were then incubated for 90 min with HIV-1 and non-adsorbed virus then removed by washing. HIV-1 infected or mock-infected PBL were then cultured in the presence of 5% recombinant interleukin-2 (IL-2) (Boehringer) and varying concentrations of the test compound. Compound-free medium was added at 4 and 8 days p.i. At 12 days p.i., p24 core antigen in the culture supernatant was analysed by a sandwich enzyme-linked immunosorbent assay ELISA (Dupont). Cytotoxicity was determined by the MTT protocol used for MT-4 cells.

§ Peripheral blood mononuclear cells were isolated by the Ficoll-Hypaque technique. The plastic-adherent cell fraction was grown for 1 week in RPMI 1640 with 20% endotoxin-free FCS (Greiner BV) and 2 mM L-glutamine and seeded in 48-well plates (Costar). Following 2 h adsorption at 37 °C of HTLV-III_{BA-L}¹³ (from M. Popovic), the cells were washed extensively, and varying concentrations of the test compound added. Cells were replenished weekly with compound-free medium. After 7 days, adherent cells (M/M) were >80% LeuM3⁺ and less than 1% Leu 1⁺, Leu 4⁺, and Leu 12⁺ (Becton-Dickinson). Neither recombinant IL-2 nor mitogen was added. Fourteen days p.i., cell cultures were freeze-thawed twice, and p24 quantified as for PBL.

|| The anti-Moloney sarcoma virus assay is described in ref. 25.

All data represent median values for at least 3 separate experiments. ND, Not determined.

In MT-4 cells, the HIV-1 strains HTLV-III_B, RF (from a patient of Haitian descent) and HE (isolated by us from a Belgian patient with AIDS) are inhibited at comparable concentrations. At 10 nM, R82150 inhibits HIV-1 p24 production of the monocytotropic HTLV-III_{BA-L} strain by 50% in fresh human monocyte/macrophage cultures. AZT and DDC show variable activity and cytotoxicity, depending on the choice of cells.

Surprisingly, R82150 (Table 2) and R82913 (not shown) are not active against the HIV-2 strains ROD and EHO, whereas AZT and DDC are equally effective against HIV-1 and HIV-2: these chemicals, then, clearly discriminate between HIV-1 and HIV-2. Although they are related, HIV-1 and HIV-2 differ in their genomic sequence, antigenic properties and in the size of

their proteins¹¹. HTLV-I protein expression in MT-2 cells¹², which is stronger than in the related MT-4 cells, is not affected by R82150 concentrations of up to 30 μM (not shown). Simian immunodeficiency virus (SIV), which is closely related to HIV-2, and murine Moloney sarcoma virus (MSV) were both insensitive to R82150, and R79882 (Table 1) was inactive against feline immunodeficiency virus (M. Koolen, personal communication), so these animal models are unsuitable for testing our TIBO derivatives. Various other RNA viruses (vesicular stomatitis, Sindbis, Semliki Forest, parainfluenza, rhino, polio, coxsackie and reovirus) and DNA viruses (herpes simplex, varicella zoster, cytomegalo, vaccinia) were also insensitive to R82150. So far, we have found no exception to the extremely selective inhibition

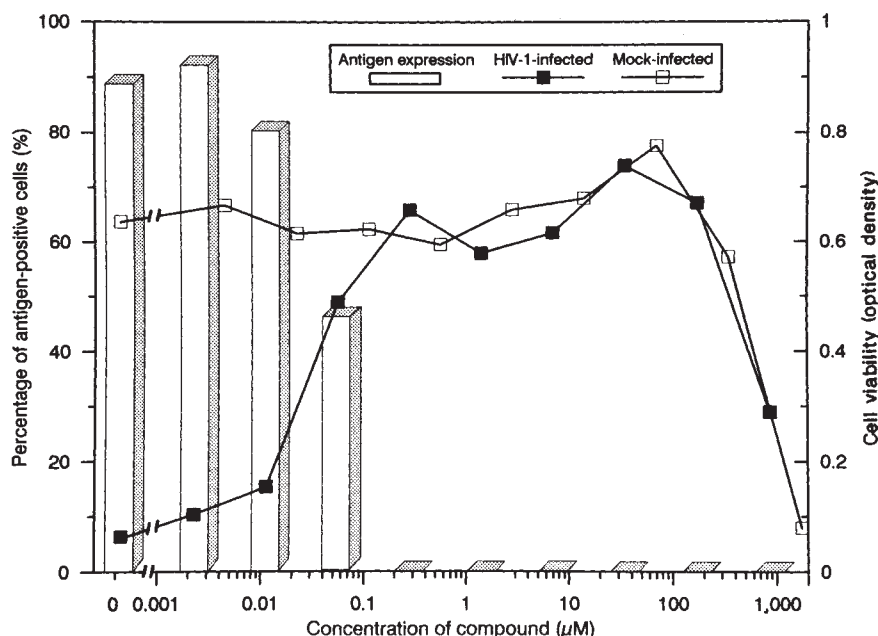
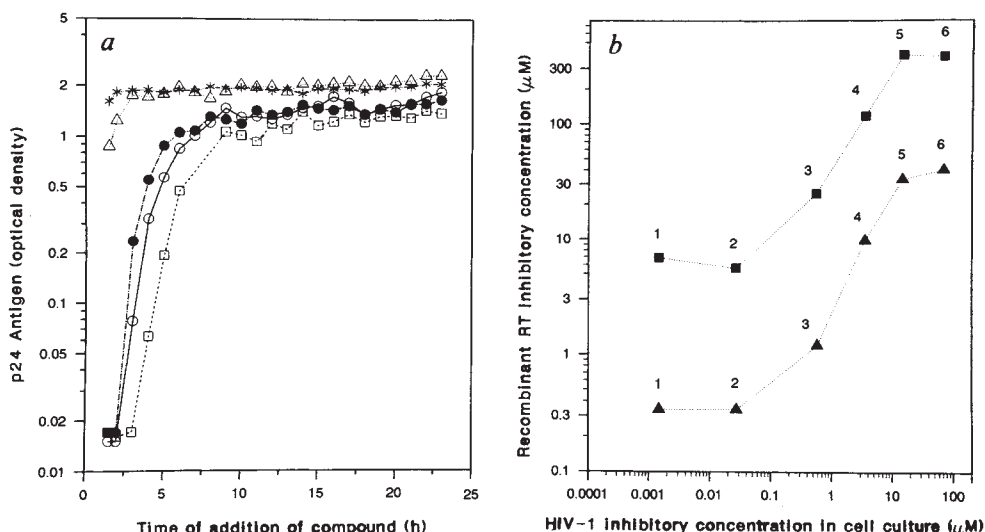


FIG. 1 Inhibitory effects of R82150 on HIV-1 antigen expression (bars) and on viability of HIV-1-infected (closed square) or mock-infected MT-4 cells (open square).

METHODS. All experiments were on MT-4 cells using the HTLV-III_B strain; assay conditions are described in the legend to Table 1. Four days after infection, HIV-1 antigens were revealed by an indirect immunofluorescence procedure using polyclonal antibodies¹⁰. The percentage of antigen-positive cells was determined by laser flow cytofluorometry.

FIG. 2 Studies on the mechanism of action of the TIBO-derivative R82150. *a*, Variation of p24 antigen concentration with time of addition of compounds. *b*, Correlation between HIV-1 inhibitory effects of a series of TIBO-derivatives in cell culture and inhibition of HIV-1 recombinant reverse transcriptase (RT) (1, R82913; 2, R82150; 3-5, other TIBO-derivatives; 6, R78305).

METHODS. *a*, MT-4 cells were infected at a multiplicity of infection >1 with HIV-1 (HTLV-III_B). After 60 min incubation at 37 °C, unadsorbed virus was removed by 3 washing steps. Pentosan polysulphate ($50 \mu\text{g ml}^{-1}$ or $16 \mu\text{M}$) (asterisk), aurointricarboxylic acid ($100 \mu\text{g ml}^{-1}$ or $237 \mu\text{M}$) (open triangle), AZT ($0.05 \mu\text{g ml}^{-1}$ or $0.187 \mu\text{M}$) (open circle), DDC ($2 \mu\text{g ml}^{-1}$ or $9.5 \mu\text{M}$) (filled circle) and R82150 ($0.5 \mu\text{g ml}^{-1}$ or $1.7 \mu\text{M}$) (open square) were added to parallel cultures in microtitre plates at different times p.i. HIV-1 core protein (p24) was determined 48 h p.i. by a sandwich ELISA. *b*, Reverse transcriptase (p66) (MicroGeneSys) is derived from an HIV-1 *pol* gene fragment which codes for all the reverse transcriptase, plus 13 amino acids of the C terminus of the protease and 34 amino acids of the N terminus of the endonuclease; it is produced in an insect cell/baculovirus expression system. The apparent molecular weight (by SDS-PAGE) is $\sim 71,000$. Final enzyme concentrations were 2 or $4 \mu\text{g ml}^{-1}$, depending on the choice of the template/primer:



poly(rA)·oligo(dT)₁₂₋₁₈ ($8 \mu\text{g ml}^{-1}$) (filled square) or poly(rC) ($40 \mu\text{g ml}^{-1}$)/oligo(dG)₁₂₋₁₈ ($1.2 \mu\text{M}$) (filled triangle). The respective substrates were [methyl-³H]thymidine 5'-triphosphate or deoxy[8-³H]guanosine 5'-triphosphate, both at a concentration of $2.5 \mu\text{M}$. The reaction mixture (50 mM Tris-HCl buffer, pH 8.4) contained 10 mM Mg^{2+} , 100 mM K^{+} , 2.2 mM dithiothreitol and 0.03% Triton X-100. Incorporation of labelled substrates was determined by a standard trichloroacetic acid precipitation procedure.

of HIV-1 replication by TIBO derivatives.

Dose-response curves for various measures of HTLV-III_B inhibition by R82150 in MT-4 cells are shown in Fig. 1. Complete protection against HIV-1 cytopathicity is achieved at 350 nM and against HIV-1 antigen expression, as measured by laser flow cytofluorometry¹⁰, at 280 nM . HIV-1 p24 production in the supernatant is inhibited by 50% at 10 nM and completely at 100 nM (data not shown). In a virus yield assay, the maximum reduction of $5 \log_{10}$ in progeny virus measurable in this system was achieved at $1.4 \mu\text{M}$. In an HIV-1 plaque assay¹³, there was a 50% plaque reduction at 60 nM , and complete reduction at 700 nM . Other TIBO derivatives act like R82150 in inhibiting different measures of HIV-1 replication (data not shown).

In considering the mechanism of action, we found that R82150 (at $35 \mu\text{M}$), unlike aurointricarboxylic acid (ATA)¹⁴, does not mask the CD4/HIV receptor on MT-4 cells. In contrast with sulphated polysaccharides¹⁶⁻¹⁹, it does not block the binding of HIV-1 on MT-4 cells, or syncytium formation between uninfected MOLT-4 cells and persistently infected HUT-78 cells; in the latter, HIV-1 antigen and progeny formation is not affected by 5 days' incubation with $3.5 \mu\text{M}$ R82150. In these experiments, R82150 behaved similarly to AZT. We confirmed that R82150 did not act at an early stage in the infectious cycle by adding compounds at different times after exposure of MT-4 cells to HIV-1 (Fig. 2a); addition of pentosan polysulphate²⁰ at 1.5 h, or of ATA at 3 h after infection had no effect. Inhibition by AZT and DDC decreased when they were added 3-4 h post-infection, but addition of R82150 could be postponed for 5 h with only a slight loss of inhibitory potency. It was possible that this difference could arise from the time taken to phosphorylate nucleoside analogues, a process that is unnecessary for the activity of the TIBO derivatives. We therefore investigated the effect of R82150 on the HIV reverse transcriptase.

Using detergent-disrupted virus particles as the enzyme preparation, we found that R82150 inhibited poly(rA)·oligo(dT)₁₂₋₁₈-directed reverse transcriptase activity by 50% at a concentration of $3.1 \mu\text{M}$ (HTLV-III_B strain) and $4.9 \mu\text{M}$ (RF strain). HIV-1 recombinant p66 reverse transcriptase was inhibited at a 50% inhibitory concentration (IC_{50}) of 5.6 or

$5.9 \mu\text{M}$, with poly(rA)·oligo(dT)₁₂₋₁₈ or poly(rI)·oligo(dC)₁₂₋₁₈, respectively as the template and primer. Interestingly, reverse transcriptase inhibition was increased 15-fold (IC_{50} , $0.34 \mu\text{M}$) when poly(rC)·oligo(dG)₁₂₋₁₈ was used as the template and primer. We found no inhibition by R82150 at concentrations of up to $350 \mu\text{M}$ on reverse transcriptase from HIV-2 (ROD strain), when we used either poly(rA)·oligo(dT)₁₂₋₁₈ or poly(rC)·oligo(dG)₁₂₋₁₈ as the template and primer. This agrees with the HIV-1 specificity of R82150 in cell culture, and contrasts with other inhibitors such as AZT, suramin and phosphonoformic acid, which are equally active against the reverse transcriptase of HIV-1 and HIV-2. A reverse transcriptase-associated process is further implicated as a target of TIBO derivatives in another experiment comparing their inhibition of HIV-1 replication in cell culture with their effect on HIV-1 recombinant p66 RT: a close correlation between the IC_{50} values is found (Fig. 2b) (Spearman rank correlation: 0.89 and 0.99 for the poly(rA)- or poly(rC)-directed RT reaction, respectively; $P < 0.05$ in each case).

The search for the TIBO derivative most suitable for clinical studies in HIV-1-infected patients is in progress. In dogs, R82150 given intravenously shows an extensive tissue distribution (volume of distribution, $V_{d\text{area}} = 10.21 \text{ kg}^{-1}$); it is eliminated after metabolism, with a terminal half-life of 3.2 h . No adverse effects are seen at plasma levels exceeding the anti-HIV-1 IC_{50} -value by 1,000-fold. In six healthy male volunteers, the absolute bioavailability upon oral intake of a 200 mg dose amounted to 31% , and plasma levels exceeding the anti-HIV-1 IC_{50} were maintained for 24 h . The elimination half-life averaged 13 h , and the $V_{d\text{area}}$ was the same as in dogs. The compound was well tolerated and no significant changes in haematological, biochemical or cardiovascular variables were observed. □

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1. Barré-Sinoussi, F. *et al. Science* **220**, 868-871 (1983).
2. Gallo, R. C. *et al. Science* **224**, 500-503 (1984).
3. Clavel, F. *et al. Science* **233**, 343-346 (1986).
4. Balzarini, J. & Broder, S. in *Clinical Use of Antiviral Drugs* (ed. De Clercq, E.) 361-385 (Martinus Nijhoff, Boston, 1988).

5. Mitsuya, H. *et al. Proc. natn. Acad. Sci. U.S.A.* **82**, 7096–7100 (1985).
6. Chu, C. K. *et al. Biochem. Pharmac.* **37**, 3543–3548 (1988).
7. Pauwels, R. *et al. J. virol. Meth.* **20**, 309–321 (1988).
8. De Clercq, E. *Antiviral Res.* **12**, 1–20 (1989).
9. Janssen, P. A. J. *et al. J. pharmacol. exp. Ther.* **244**, 685–693 (1988).
10. Pauwels, R. *et al. J. virol. Meth.* **16**, 171–185 (1987).
11. Clavel, F. & Guétard, D. *AIDS* **1**, 135–140 (1987).
12. Harada, S., Koyanagi, Y. & Yamamoto, N. *Science* **229**, 563–566 (1985).
13. Nakashima, H. *et al. J. virol. Meth.* **26**, 319–330 (1989).
14. Schols, D., Baba, M., Pauwels, R., Desmyter, J. & De Clercq, E. *Proc. natn. Acad. Sci. U.S.A.* **86**, 3322–3326 (1989).
15. Dalgleish, A. G. *et al. Nature* **312**, 763–767 (1984).
16. Baba, M. *et al. Proc. natn. Acad. Sci. U.S.A.* **85**, 6132–6136 (1988).
17. Mitsuya, H. *et al. Science* **240**, 646–649 (1988).
18. Schols, D., Baba, M., Pauwels, R. & De Clercq, E. *J. AIDS* **2**, 10–15 (1989).
19. Schols, D., Pauwels, R., Baba, M., Desmyter, J. & De Clercq, E. *J. gen. Virol.* **70**, 2397–2408 (1989).
20. Baba, M. *et al. Antivir. Res.* **9**, 335–343 (1988).
21. Starcich, B. R. *et al. Cell* **45**, 637–648 (1986).
22. Clavel, F. *et al. Nature* **324**, 691–695 (1986).
23. Rey, M. *et al. Virology* **173**, 258–267 (1989).
24. Franchini, G. *et al. Nature* **328**, 539–543 (1987).
25. Balzarini, J. *et al. Biochem. biophys. Res. Commun.* **145**, 269–276 (1987).

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Attenuation of Mengo virus through genetic engineering of the 5' noncoding poly(C) tract

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THE murine cardioviruses, such as the Mengo and encephalomyocarditis viruses, and the bovine aphthoviruses, such as foot-and-mouth disease virus, are distinguished among positive-strand RNA viruses by the presence of long homopolymeric poly(C) tracts within their 5' noncoding sequences. Although the specific lengths (60–350 bases) and sequence discontinuities (for example, uridine residues) that sometimes disrupt the homopolymer have served to characterize natural viral isolates, the biological function of the poly(C) region has never been clear. We now report that complementary DNA-mediated truncation of the Mengo virus poly(C) tract dramatically attenuates the pathogenicity of the virus in mice. Animals injected with viruses with short tracts not only survived inoculation of up to 50 µg live virus (10^{11} plaque-forming units) but consistently produced high titres of neutralizing antibodies, which conferred long-term immunogenic protection from (normally) lethal virus challenge. We propose that analogous synthetic strains of foot and mouth disease virus could serve as the basis for new attenuated vaccines.

We have described complementary DNA clones containing full-length infectious Mengo virus sequences, although with shorter poly(C) tracts than the $C_{50}UC_{10}$ tract of the original parental virus¹. Plasmid pM18, for example, contained a tract of C_8 , and plasmid pM16 contained a tract of $C_{13}UC_{10}$ (Fig. 1). We have now isolated a new plasmid, pMwt, which contains the same $C_{50}UC_{10}$ tract as wild-type Mengo virus, and is the first reported complementary DNA with a parental-length poly(C) from any cardio- or aphthovirus. Because all infectious clones were derived from plasmid pM16 through direct substitution of a unique 5'-region restriction fragment, the pM plasmids differ from each other only in the lengths of their viral poly(C) tracts.

RNA transcripts from plasmid pMwt, like those from our other constructions, were fully infectious to HeLa cells, and the

virus isolated as a consequence of transfection (that is, progeny virus) was indistinguishable from parental Mengo virus for all growth characteristics in tissue culture. For all of the plasmid pM isolates, the progeny virus poly(C) tracts were precisely those cloned into cDNA, and their individual lengths and discontinuities passed with fidelity in tissue culture¹. These results show clearly that the long segments of natural cardioviral poly(C) tracts are not important for the reproductive cycle of the virus. Why then are poly(C) tracts maintained in wild-type isolates if they are dispensable for most infectious functions?

To assess whether a poly(C)-dependent phenotype is evident in the natural host, we inoculated mice with our viruses. Both encephalomyocarditis ($C_{115}UCUC_3UC_{10}$) and Mengo viruses are neurotropic, and normally cause a rapid and lethal meningo-encephalomyelitis after intraperitoneal (i.p.) or intracerebral (i.c.) injection^{2,3}. As expected, the wild-type viruses induced clear symptoms of neurological illness within the first week post-inoculation (PI), and all mice were dead by day 10. Surprisingly however, only a few of the animals receiving similar doses (i.c.) of the short poly(C) viruses became sick and died, and of these, none exhibited overt symptoms of disease (hind-leg paralysis, hunched posture and ataxia) until almost one week PI. The median lethal dose (LD_{50} ; i.c.) for pM16 or pM18 progeny virus was about 10^6 times higher than that for parental Mengo virus, or for pMwt progeny virus, for which genetically engineered restoration of poly(C) length resulted in unambiguous loss of the attenuated phenotype and reacquisition of pathogenicity (Fig. 2).

The remarkable degree of attenuation for the short-tract viruses was especially apparent in i.p. studies, in which it proved difficult to establish a true upper limit for the lethal dose. All mice receiving more than 10^{10} plaque-forming units (PFU; i.p.) of pM16 or pM18 progeny virus survived the experiments without exhibiting disease. Although a few animals receiving

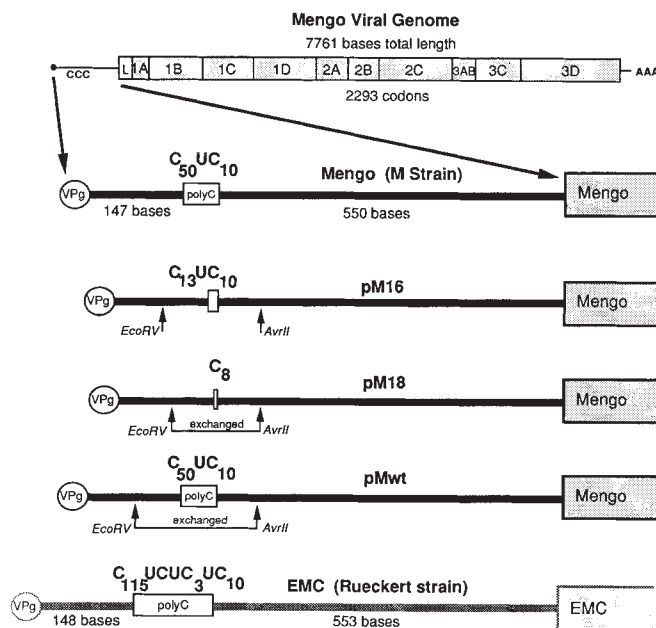


FIG. 1 5' noncoding regions of wild-type and progeny cardioviruses. Representation shows relative lengths of poly(C) tracts and origin of sequences for genetically engineered progeny viruses and parental Mengo and encephalomyocarditis (EMC) viruses. All pM plasmids, including pMwt, were derived from plasmid pM16 by exchange of the *EcoRV*–*AvrII* cassette as described in ref. 1. Polyprotein nomenclature is according to ref. 9. Base sequence numbers are according to G.M.D. and A.C.P., manuscript in preparation.

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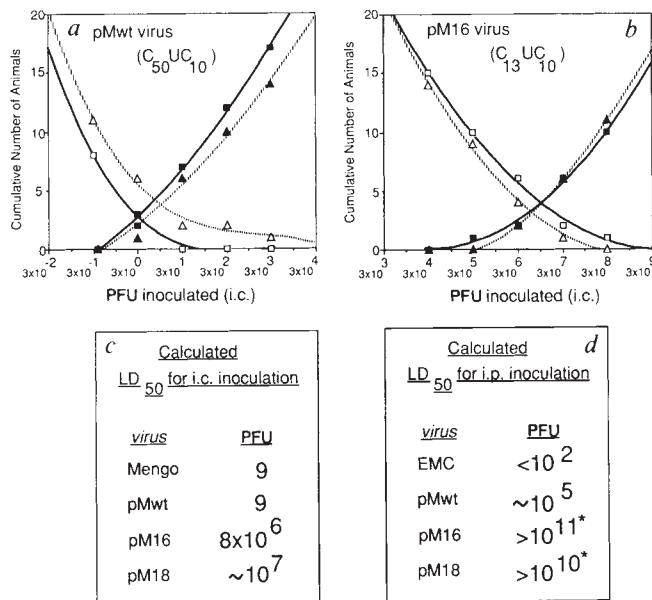


FIG. 2 LD₅₀ determinations. Groups of five animals (4.5-week-old Swiss mice) were inoculated i.c. on day 1 with the indicated doses (PFU) of pMwt (a), or pM16 (b) progeny virus. On day 14 PI, the survivors of each dose, in addition to the survivors of all higher doses, were tabulated, and are plotted as the cumulative number of surviving animals for that dose (open symbols). Likewise, the cumulative number of dead animals (filled symbols) represents the sum of the nonsurvivors of that dose, in addition to nonsurvivors of all lower doses. The mean time of death was generally between days 5–8 for all tested isolates, and there were no further deaths at any dose after day 11. Separate determinations with male (squares) and females (triangles) are presented as examples. Data for the Mengo and pM18 viruses, and the i.p. values, were collected in a similar manner but are not shown. LD₅₀ calculations (c, d) were by the method of Reed and Muench¹⁰, and are averaged values for male and females. Asterisks * indicate minimum estimated values (see text).

higher doses (10^{11} PFU per animal) died after their inoculations, we believe, from the rapidity with which they succumbed (within the first day PI), that their deaths were due to toxic shock from the high dose of foreign protein (representing $> 50 \mu\text{g}$ of live virus), rather than infection with Mengo virus.

Characteristically, the brains of animals which died after inoculation with pMwt progeny, Mengo or EMC virus contained high titres of recoverable virus (10^6 – 10^7 PFU per ml of brain suspension). Similar titres were also recoverable from animals receiving the short-tract viruses whenever the initial inoculum

was potent enough to cause mortality (for example, $> 10^7$ PFU i.c.). Virus isolated from these mice has never shown evidence of poly(C)-length reversion, heterogeneity or diminished attenuation, but we are continuing to passage isolates serially through brains to determine if a reversion can be forced. In animals receiving non-fatal doses, the attenuated viruses showed only limited replication (measured after i.c. injection), and even at doses near the LD₅₀, the brain titres were at least a factor of 10 lower than those reached in equivalent pMwt-progeny-virus infections (Fig. 3). Viral clearance was most obvious during the second week PI, and no infectious virus was ever isolated from any animal after day 21.

Dramatic biological effects from 5'-region changes are not without precedent in picornaviruses. A single noncoding nucleotide has been proposed as a primary determinant of neurovirulence in polio, for example⁴. Its consequences seem to be affected through a translational block evident only during growth in particular cells. But poliovirus does not contain a poly(C) tract, and the culpable poliovirus nucleotide lacks direct sequence or structural analogy in cardioviruses^{5,6}. Thus, the mechanism for poly(C)-mediated attenuation remains unknown. Whether the short-tract strains are simply more effective at inducing an important protective element within the host's defence system, or whether they are defective in some critical step within a specific target tissue, remains to be determined. We also do not yet know if the attenuation is actually correlated in a progressively linear or quantum-type relationship with increasing poly(C) length, or even whether the peculiar strain-specific sequence discontinuities play a part in this phenomenon.

Whatever the mechanism of attenuation however, it is clear that in all the experiments described above, sub-lethal doses of short-tract viruses served as excellent immunogens for mice. Inoculated animals consistently developed 10^3 – 10^4 more neutralizing antibody titre than did control animals within the first week PI (Fig. 4). Challenge (usually at day 30 PI) with EMC virus at a dose (10^4 PFU) determined to be at least 100 times that of the LD₅₀ for non-immune animals (refer to Fig. 2) gave a 100% survival rate, without any symptoms of disease. Apparently, in spite of their poor replication rate, the attenuated viruses were effectively recognized by the mouse immune system and seemed to have protectively vaccinated any animal receiving a sublethal dose. Some of our animals have now been followed for more than one year after their initial inoculations and show only a small decrease in neutralizing titre. They are still protected against lethal virus challenge and we have every expectation that they have been effectively immunized for life.

Within the relatively simple parameters of our pathogenicity assay (death), we cannot unequivocally say that sublethal doses of attenuated viruses had no adverse effects on animals, but survivors generally behaved in the robust manner of control

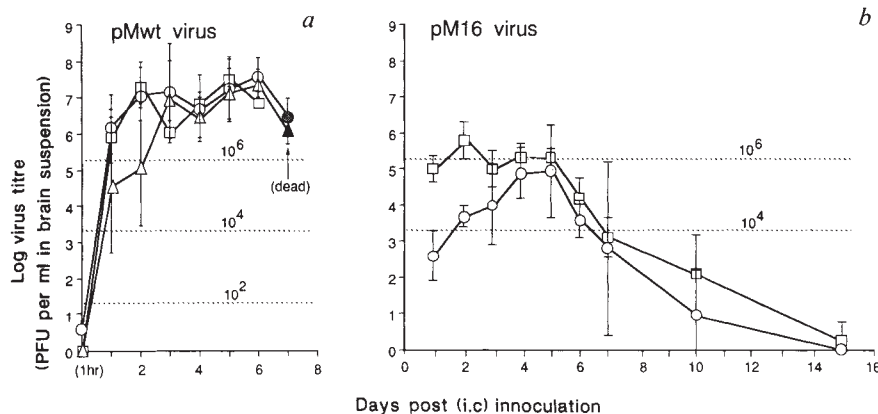


FIG. 3 Amount of viruses recovered from the brains of inoculated animals. Groups of 45 female mice were inoculated i.c. into the left (a) hemisphere with 10^2 (triangles), 10^4 (circles) or 10^6 (squares) PFU of pMwt progeny virus or with 10^4 or 10^6 PFU of pM16 progeny virus (b). On the indicated days PI, five animals from each group were killed and brain samples (left hemisphere) analysed for infectious virus. Day 7 pMwt-progeny-virus samples (dark symbols) were from dead animals, as there were no survivors. Data are plotted as $\log_{10}$ of PFU ml⁻¹ detectable in 10% brain suspensions for the average of five animals. The standard deviation for each value is shown. Horizontal lines represent the theoretical maximum recovery of all inoculum PFU, assuming no loss owing to clearing, degradation or isolation methods.

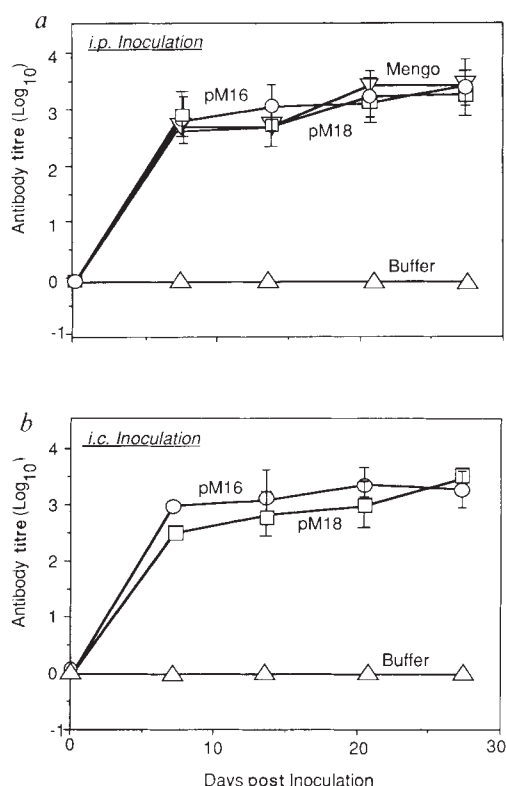


FIG. 4 Serum antibody titres in inoculated animals. Groups of five animals were inoculated i.c. (10^6 PFU) or i.p. (10^7 PFU) with pM16 progeny virus ($C_{13}UC_{10}$, $\circ$), pM18 progeny virus (C_8 , $\square$), Mengo virus ($C_{50}UC_{10}$, ∇) or PBSA ($30 \mu\text{l}$ i.c., $100 \mu\text{l}$ i.p., $\triangle$). On the indicated days, surviving animals were bled from the retroorbital sinus and serum titres for Mengo virus antibodies determined in microneutralization assays¹¹. Data are plotted as the minimum effective dilution of serum ($\log_{10}$) to confer complete protection to a HeLa cell monolayer from 10^4 PFU of Mengo virus. All assays were carried out in duplicate and each point is an averaged determination for 2–5 animals. Error bars, s.d.

animals and many have lived extended and apparently healthy lives long after the experiments. Although we are now carrying out comprehensive histopathology studies to measure quiescent changes in disease aetiology, we already suspect that the primary phenotypic manifestation of the altered poly(C) tracts is solely the altered dose response relative to that of the wild-type strains. If this conclusion proves correct, the observations reported here are especially relevant to work with the related foot-and-mouth disease (FMD) virus, which also has a 5' noncoding poly(C) tract similarly disposed within its genome. Previous results from field-isolated or laboratory strain FMD virus have conflicted regarding the potential correlation between poly(C) length and aphthovirus pathogenicity^{7,8}. That all previously characterized 'short'-tract FMD virus strains contained a poly(C) tract at least twice as long as that of the completely pathogenic Mengo or pMwt progeny virus used here, would explain the discrepant results. We suggest that the potent attenuation observed with plasmid pM progeny viruses could also be a property of genuinely analogous FMD virus strains carrying truly short (for example, C_8 to C_{20}) homopolymeric tracts. We hope that cDNA-derived FMD isolates comparable to those that we have developed for the Mengo virus will eventually form the basis for new attenuated vaccines, capable of conferring effective immunogenic protection in the same way that the pM progeny viruses successfully protected mice in the study described here. $\square$

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1. Duke, G. M. & Palmenberg, A. C. *J. Virol.* **63**, 1822–1826 (1989).
2. Guthke, R., Ckenstedt, A. V., Güttner, J., Stracke, R. & Bergter, F. *Acta. Virol.* **31**, 307–320 (1988).
3. Veckenstedt, A. *Acta. Virol.* **18**, 501–507 (1974).
4. La Monica, N. & Racaniello, V. R. *J. Virol.* **63**, 2357–2360 (1989).
5. Pilipenko, E. V., Blinov, V. M., Chernov, B. K., Dmitrieva, T. M. & Agol, V. I. *Nucleic Acids Res.* **17**, 5701–5711 (1989).
6. Pilipenko, E. V. *et al. Virology* **168**, 201–209 (1989).
7. Harris, T. J. R. & Brown, F. *J. gen. Virol.* **34**, 87–105 (1977).
8. Costa Giomi, M. P. *et al. Virology* **162**, 58–64 (1988).
9. Rueckert, R. R. & Wimmer, E. *J. Virol.* **50**, 957–959 (1984).
10. Reed, L. J. & Muench, H. A. *Am. J. Hyg.* **27**, 493–497 (1938).
11. Sherry, B. & Rueckert, R. R. *J. Virol.* **53**, 137–143 (1985).

Localization of VP4 neutralization sites in rotavirus by three-dimensional cryo-electron microscopy

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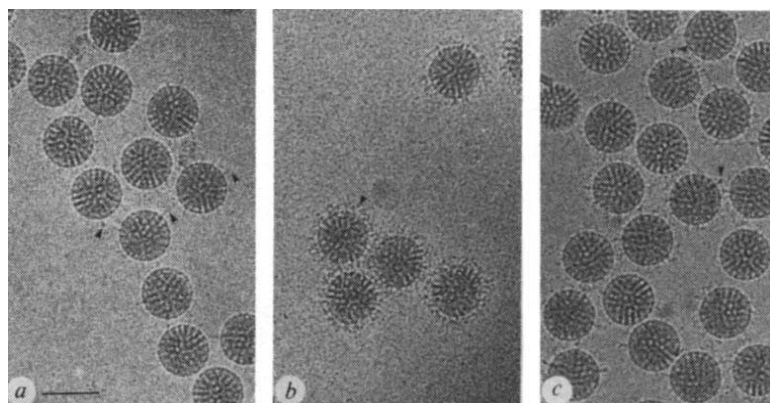
THREE-DIMENSIONAL structures of several spherical viruses have been determined by electron microscopy^{1–6} and X-ray crystallography^{7,8}. We report here the first three-dimensional structure of the complex between an intact virus and Fab fragments of a neutralizing monoclonal antibody. The antibody is against VP4, one of the two outer capsid proteins of rotaviruses. These large icosahedral viruses cause gastroenteritis in children and young animals and account for over a million human deaths annually⁹. VP4 in these viruses has been implicated in several important functions such as cell penetration, haemagglutination, neutralization and virulence^{10–12}. Here we demonstrate that the surface spikes on rotavirus particles are made up of VP4. Antigenic sites are located near the distal ends of the spikes and two Fab fragments bind to each of the sixty spikes. The mass of the spike indicates that it is a dimer of VP4. The bilobed structure at the distal end of the spike may be involved in both the attachment to the cell and in viral penetration. A novel feature in the virus–Fab complex is the structural difference between the two chemically equivalent Fab fragments on each spike, which could be indicative of variations in the Fab elbow angles.

Cryo-electron micrographs of native double-shelled virions embedded in vitreous ice (Fig. 1a) show the characteristic smooth outer margin of the virions and the spikes emanating from them⁴. Cryo-electron micrographs of virions complexed with anti-VP4 IgGs (Fig. 1b) show additional density at the surface of the virions, indicating binding of the IgG molecules. What is not clear from these micrographs is whether the IgG molecules are interacting with the viral surface or the spikes. Because of the bivalent nature of IgGs, virions are often aggregated and such aggregation makes a three-dimensional reconstruction difficult. However, this micrograph confirms the feasibility of visualizing antibody–virus complexes by cryo-electron microscopy. The problem of aggregation is avoided by using Fab fragments of IgG. Although virus–Fab complex formation is visually not as dramatic as with IgGs, careful inspection of micrographs of virus–Fab complexes (Fig. 1c) shows an increase in the density at the distal ends of the spikes. Apart from this, no other marked differences between the native and Fab-bound virions are evident. We have determined the three-dimensional

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FIG. 1 Cryo-electron micrographs of *a*, native rotavirus, *b*, rotavirus complexed with anti-VP4 IgGs and *c*, rotavirus complexed with Fab fragments from the IgG used in *b*. In *a*, arrows denote protein spikes; in *b*, they denote IgGs; and in *c*, they denote Fab-bound regions on the spikes. Scale bar, 1,000 Å. The individual spikes on the virus particles appear very faint. The spike density, as shown by the arrows in *a* and *c*, becomes more visible when two spikes superimpose in projection.

METHODS. The specimen in these micrographs was embedded in a thin layer of ice across a hole of a holey carbon grid. The specimen preparation method was as described previously^{4,13}. Micrographs were recorded using electron doses of 4–5 e per Å² on the specimen at a magnification of 30,000 with an underfocus of ~3 µm on a JEOL1200 microscope at -150 °C. SA11 strain (clone 3) of rotavirus was purified from infected MA104 cells as described earlier²⁴. SDS-polyacrylamide gel analysis confirmed that VP4 was intact and not cleaved. Neutralizing monoclonal antibody (2G4) raised against VP4 was used^{25,26}. Fab fragments were obtained from purified IgGs by overnight digestion with papain in the presence of 0.01 M dithiothreitol and 2 mM EDTA, pH 7.0. After termination of the reaction with iodoacetamide, Fc fragments and undigested antibody molecules were removed by adsorption onto formalin-fixed *Staphylococcus aureus* and the Fab fragments dialysed against 0.01 M phosphate buffer, pH 7.2. The Fab



samples were analysed by PAGE to check that digestion was complete and the preparation pure. Purified IgG and Fab fragments both neutralized virus infectivity when tested in plaque reduction neutralization assays with 100 plaque forming units of virus²⁶. Virus particles were incubated overnight at 4 °C with an excess of either IgG or Fab in 20 mM Tris-buffered saline before cryo-electron microscopy.

structure of both native and Fab-virus complexes from such micrographs using computer image processing techniques to a nominal resolution of 35 Å.

The three-dimensional structure of the native rotavirus shows the characteristic outer surface perforated by 132 channels at all the six- and five-coordinated centres on a $T=13I$ icosahedral lattice (Fig. 2*a*). In this reconstruction we see the entire length of the spikes and also several of their structural features. These spikes are ~120 Å long and are situated at the outer edge of the type II channels surrounding the icosahedral fivefold axes⁴. Each virion has sixty such spikes. From the surface of the virus up to a height of 45 Å, the spikes are thin, with a maximum width of ~35 Å. Beyond this point, there is a well defined globular domain of ~55 Å in diameter. Lying across the top of this globular domain is a bilobed structural feature, 40 Å wide and 70 Å across. Earlier, we interpreted the spike as being shorter⁴ because the reconstructed map tended to be noisy beyond this length, but here we have increased the number of particles in the reconstruction, followed by a refinement of the orientations of each particle with respect to the entire data, to

reduce the noise level. The dimensions of the channels and other structural features in this reconstruction are identical to those described previously⁴, despite minor differences (as shown by RNA patterns) in the SA11 strains.

The three-dimensional structure of the Fab-bound virus (Fig. 2*b*) shows a significant increase in the mass density near the distal ends of the spikes. Other structural details remain unchanged in comparison with the native structure. The extra mass density that is seen in this reconstruction is almost perpendicular to the length of the spikes, with a thickness and length of about 35 Å and 170 Å respectively. This extra density is due to the Fab. To define the regions belonging to the Fab and the spike protein, we determined the difference between the native and Fab-bound structures. In this difference map, the density that should correspond to Fab is consistently split into two parts (Fig. 3). Each part has dimensions of about 65 Å × 40 Å × 35 Å and the two parts are separated by ~40 Å. The distal end of the spike fits into the region separating the two parts.

The calculated relative molecular mass (M_r) and dimension of each of the two parts of extra density on every spike agree

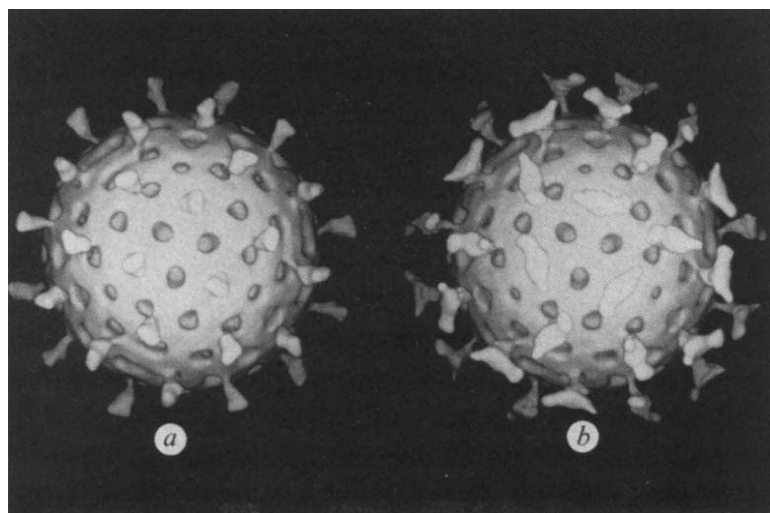


FIG. 2 Surface representations of the three-dimensional structures of *a*, the native virus and *b*, Fab-virus complex, along the icosahedral 3-fold axes. Only the upper hemisphere is displayed for clarity.

METHODS. The chosen electron micrographs were digitized with a Perkin Elmer microdensitometer 1010M with scanning interval of 8.3 Å in the object. The virus particles for processing were selected by eye and boxed into 128 × 128 pixel areas. They were centred in the box using cross-correlation methods. The orientation of each particle relative to the icosahedral symmetry axes was determined using the common lines method²⁷. Once the orientations of the particles were determined, the phase origins of the particles were refined. The orientation of each particle was then refined with respect to the entire data using cross common lines. The mean phase residuals for the data up to 35 Å was 40° (±4°) for the native and 41° (±4°) for the complex particles. The three-dimensional structure was reconstructed from the images using cylindrical expansion methods²⁷. Thirty particles were used in both reconstructions. The defocus values of the images permitted reconstruction of the structure to 35 Å, with no corrections for the phase reversal due to the contrast transfer function of the microscope.

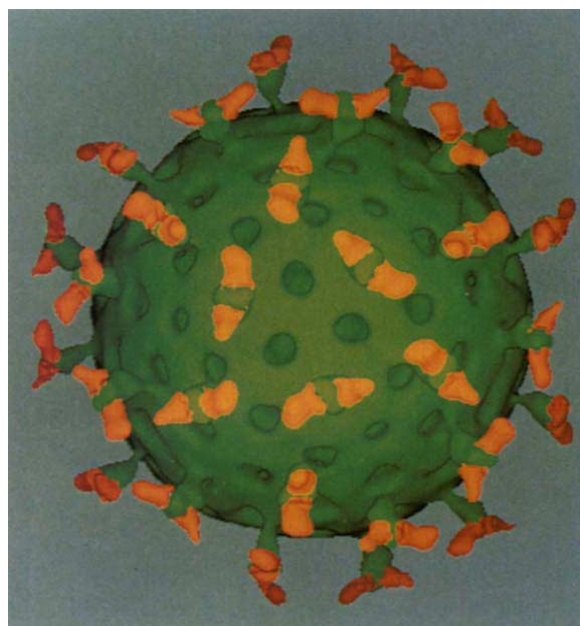


FIG. 3 Surface representation of the Fab-virus complex indicating the Fab fragments (yellow) with respect to the native structure (green). The mass density corresponding to the Fab fragments was determined by computing the difference between the native and the complex structures²⁸. The density values have been contoured at a level of 1.4 standard deviations from the mean level in the map displayed here, and also in those in Fig. 2. The peak density in the regions assigned to Fab was at 3.5 standard deviations. In the original maps, the peak density was at 4.0 standard deviations. Small disconnected regions of surface density in the difference map have been removed to show the main features more clearly. These regions had a maximum contour level of 1.5 standard deviations.

with those of a single molecule of Fab ($M_r \approx 50,000$). This indicates that there are two Fabs interacting with each spike. As the Fabs are monoclonal to VP4, these results confirm our prediction that the spike protein is VP4 (ref. 4). The calculated M_r of the entire spike is 120,000, which is significantly higher than that predicted ($M_r = 88,000$) for a single molecule of VP4 (ref. 13). In these calculations, an average protein density of 1.30 g ml^{-1} is assumed; they also depend on the choice of the contour level, but because of the sharp contrast between the protein and solvent (ice) in the images, that choice is restricted. When we selected a contour level such that mass of the spike equalled 88,000, the spike was seen to be disconnected from the surface by $\sim 50 \text{ \AA}$. Furthermore, the actual mass of the spike is likely to be more than the estimated value of 120,000. Because the 'white rings' seen around the particles, due to underfocusing, would affect the weight with which the spikes are seen in the reconstruction, the portion of the spikes close to the capsid appear thinner than they actually are. Thus, our calculations strongly indicate that the spike is a dimer of VP4, perhaps with some of the remaining mass embedded inside the surface of the virion.

A closer look at the two Fab molecules on each spike shows recognizable differences, particularly at their free ends (Fig. 4). The two Fab fragments, although they are chemically identical, have different local environments because of their location on the icosahedral lattice. Thus, they are not forced by symmetry constraints to show structural similarity. A well known variable

in Fab structures is the so-called elbow angle describing the angle between the local axes of symmetry in the V and C modules¹⁴. In the various high-resolution structures of free Fabs¹⁴ and Fab-antigen complexes^{15,16}, this angle varies from 130° to 180° . A plausible explanation for the observed structural difference between the two Fab fragments bound to each spike is that they exhibit different elbow angles.

These results demonstrate that the 60 surface spikes of rotavirus that extend distally to a length of $120 \text{ \AA}$ are made up of VP4. The mass of the spike, and the fact that two Fab fragments bind to each spike, indicates that it is a VP4 dimer. In many strains of rotavirus, VP4 is a haemagglutinin¹⁰. The only viral haemagglutinin whose crystal structure is known is that of influenza virus¹⁷. The overall length of the influenza haemagglutinin trimer is $135 \text{ \AA}$, which is similar to that of the VP4 dimer. Recently it has been shown that baculovirus-expressed VP4 can haemagglutinate (ref. 18; and M.K.E. and S. E. Crawford, unpublished data), which implies that VP4 is oligomeric, but as yet there is no direct evidence that VP4 is a dimer.

VP4 in rotaviruses is susceptible to proteases: trypsin cleaves VP4 into VP5* ($M_r = 60,000$) and VP8* ($M_r = 28,000$), resulting in increased viral infectivity¹⁹ and enhanced internalization of infectious rotavirus, a process that is inhibited by neutralizing antibodies^{20,21}. The monoclonal antibody (2G4) that we used here binds to the VP5* region of VP4 (ref. 20), and both the IgG and Fab fragments used in this study neutralize virus infectivity. We infer that the distal end of the spike contains the

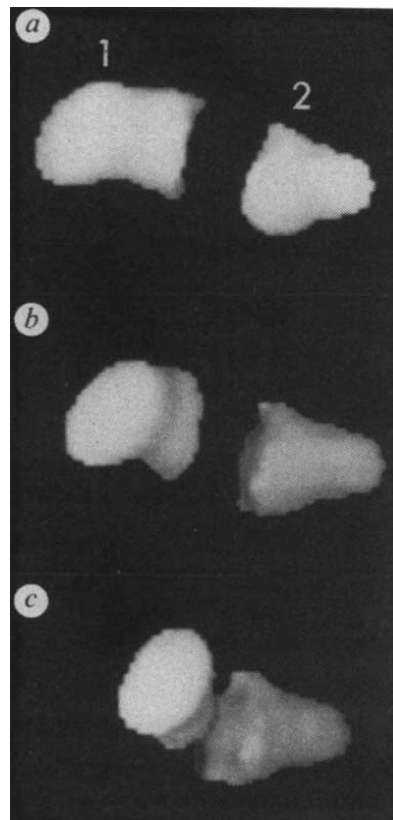


FIG. 4 Isolated views of the two Fabs, denoted by 1 and 2, attached to a spike. For clarity the density corresponding only to Fabs are shown. *a*, View looking down along the length of the spike. In this view, Fab 1 has its long axis lying parallel to the plane of the paper and almost perpendicular to the length of the spike, whereas Fab 2, pointing upwards from the plane of the paper, makes an angle of $\sim 120^\circ$. *b*, View in which *a* has been rotated so that the long axis of Fab 2 lies parallel to the plane of the paper, allowing a direct comparison of the shape of Fab 2 with Fab 1 in *a*. *c*, View in which *a* has been further rotated to expose the region of Fab 2 that is in direct contact with the spike.

VP5* region. The amino-acid sequence in this neutralization region is largely hydrophobic, having similarities to putative fusion regions of Sindbis and Semliki Forest viruses; this region might therefore participate in viral penetration²⁰.

The localization of VP4 to the rotavirus spikes suggests involvement of this protein in the viral entry process, including cell attachment. But others have proposed that VP7 is the cell-attachment protein²¹⁻²³. We consider that VP4 is the more likely candidate because it exhibits haemagglutination activity both in the free state¹⁸ and when bound to virus¹⁰. We propose that the exposed region of the distal end of the spike is involved in the initial attachment to the cell and the Fab binding region is involved in cell penetration. A plausible mechanism by which the antibody used here neutralizes infectivity is to block this region, which is critical for viral entry into the cell. □

Received 1 September; accepted 5 December 1989.

1. Crowther, R. A. & Klug, A. A. *Rev. Biochem.* **44**, 161-182 (1975).
2. Vogel, R. H., Provencher, S. W., von-Bonsdorff, C.-H., Adrian, M. & Dubochet, J. *Nature* **320**, 533-535 (1986).
3. Fuller, S. D. *Cell* **48**, 923-934 (1987).

4. Prasad, B. V. V., Wang, G. J., Clerx, J. P. M. & Chiu, W. *J. molec. Biol.* **199**, 269-275 (1988).
5. Baker, T. S., Drak, J. & Bina, M. *Proc. natn. Acad. Sci. U.S.A.* **85**, 422-426 (1988).
6. Schrag, J. D., Prasad, B. V. V., Rixon, F. J. & Chiu, W. *Cell* **56**, 651-660 (1989).
7. Rossmann, M. G. & Reuckert, R. R. *Microbiol. Sci.* **4**, 206-214 (1987).
8. Acharya, R. *et al. Nature* **337**, 709-716 (1989).
9. De Zoysa, I. & Feachem, R. G. *Bull. WHO* **63**, 569-583 (1985).
10. Estes, M. K. & Cohen, J. *Microbiol. Rev.* **53**, 410-449 (1989).
11. Kapikian, A. Z. & Chanock, R. M. in *Virology* (ed. Fields, B. N.) 1353-1404 (Raven, New York, 1990).
12. Kaljot, K. T., Shaw, R. D., Rubin, D. H. & Greenberg, H. B. *J. Virol.* **62**, 1136-1144 (1988).
13. Dubochet, J. *et al. Q. Rev. Biophys.* **21**, 129-228 (1988).
14. Davies, D. R. & Metzger, H. A. *Rev. Immun.* **1**, 87-117 (1983).
15. Amit, A. G., Marriuzza, R. A., Phillips, S. E. V. & Poljak, R. J. *Science* **233**, 747-753 (1986).
16. Colman, P. M. *et al. Nature* **326**, 358-363 (1987).
17. Wilson, I. A., Skehel, J. J. & Wiley, D. C. *Nature* **289**, 366-373 (1981).
18. Mackow, E. R., Barnett, J. W., Chan, H. & Greenberg, H. B. *J. Virol.* **63**, 1661-1668 (1989).
19. Estes, M. K., Graham, D. Y. & Mason, B. B. *J. Virol.* **39**, 879-888 (1981).
20. Mackow, E. R. *et al. Proc. natn. Acad. Sci. U.S.A.* **85**, 645-649 (1988).
21. Fukuhara, N., Yoshie, O., Kitaoka, S. & Konno, T. *J. Virol.* **62**, 2209-2218 (1988).
22. Matsuno, S. & Inouye, S. *Infection and Immunity* **39**, 155-158 (1983).
23. Sabara, M., Gilchrist, J. E., Hudson, G. R. & Babiuk, L. A. *J. Virol.* **53**, 58-66 (1985).
24. Mason, B. B., Graham, D. Y. & Estes, M. K. *J. Virol.* **46**, 413-423 (1983).
25. Shaw, R. D., Vo, P. T., Offit, P. A., Coulson, B. S. & Greenberg, H. B. *Virology* **155**, 434-451 (1986).
26. Burns, J. W., Greenberg, H. B., Shaw, R. D. & Estes, M. K. *J. Virol.* **62**, 2164-2172 (1988).
27. Crowther, R. A. *Phil. Trans. Roy. Soc. Lond B* **261**, 221-230 (1971).
28. Vigers, G. P. A., Crowther, R. A. & Pearse, B. M. F. *EMBO J.* **5**, 2079-2085 (1986).

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Turnover of fluorescently labelled tubulin and actin in the axon

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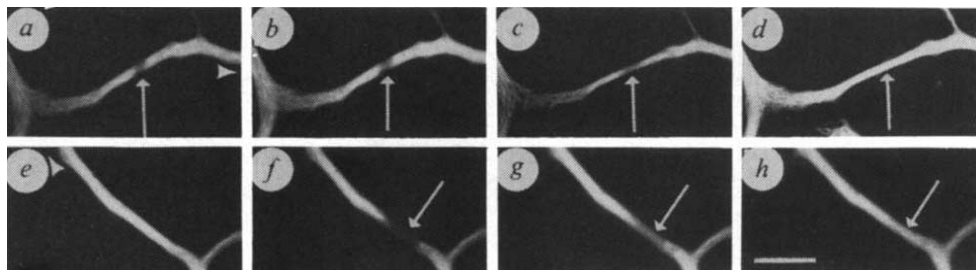
THE cytoskeleton has an important role in the generation and maintenance of the structure of the axon^{1,2}. Microtubules, neurofilaments and actin, together with various kinds of associated proteins, form highly organized dynamic cytoskeletal structures²⁻⁴. Because tubulin and actin molecules are essential cytoskeletal components² and are transported down the axon^{5,6}, it is important to understand their dynamic behaviour within the axon. Although previous pulse-labelling studies have indicated that the axonal cytoskeleton is a static complex travelling down the axon⁶, this view has been challenged by the results of several recent experiments⁷⁻¹⁴. We have now addressed this question by analysing the recovery of fluorescence after photobleaching fluorescent analogues of tubulin and actin in the axons of cultured neurons.

We did not observe movement or spreading of bleached zones along the axon, both in neurons injected with fluorescein-labelled tubulin and actin. All bleached zones recovered their fluorescence gradually, however, indicating that microtubules and actin filaments are not static polymers moving forward within the axon, but are dynamic structures that continue to assemble along the length of the axon.

We injected cultured dorsal root ganglion (DRG) cells with fluorescein-labelled tubulin and actin. Low-light level video microscopy enabled us to obtain sequential images of the fluorescence in the axon. To bleach a region of the axon, we applied an intense laser-beam pulse. Although prolonged exposure (>2 s) of microtubules to a laser beam results in their gradual destruction¹⁵, a very short application of a focused laser beam (<10 ms) did not cause their breakdown (Fig. 1d), nor did it affect their dynamic behaviour (Fig. 2). Continuous illumination of cells with a mercury arc lamp for >1 min, a procedure previously used for photobleaching¹⁶, frequently led to the arrest of axonal growth (data not shown). We did not, therefore, use this bleaching method.

When cells were injected with fluorescein-labelled tubulin and then bleached by a laser beam, the bleached zone remained at

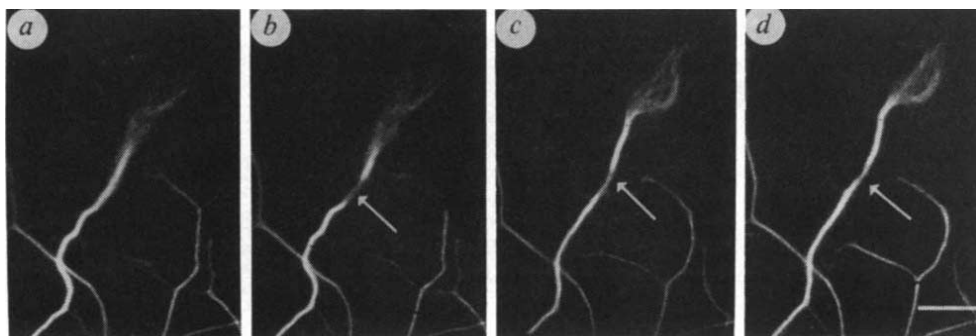
FIG. 1 Fluorescence recovery after photobleaching of fluorescein-labelled tubulin in the axons of DRG neurons. a-d, Two frames of video microscopy showing an axon immediately after (a) and 21 min after (b) photobleaching, and micrographs of the same axon permeabilized and fixed 60 min after photobleaching showing the images of fluorescein-labelled microtubules (c) and total microtubules detected by rhodamine anti-tubulin immunofluorescence (d). The bleached zone (arrows in a-c) remained in the same position up to 1 h after photobleaching and anti-tubulin immunofluorescence revealed that bleached microtubules were intact (arrow in d). e-h, Four successive frames of video microscopy before (e), immediately after (f), 30 min after (g) and 90 min after (h) photobleaching. The bleached region (arrows in f-h) recovered its fluorescence prominently 90 min after photobleaching. Arrowheads indicate the direction of the neurite tips. Bar, 10 μ m.



METHODS. Tubulin was labelled with 5(6)-carboxy-fluorescein succinimidyl ester²⁸. DRG neurons were isolated from adult mouse with density gradients²⁹ and plated onto laminin-coated glass coverslips. Cells were injected 5-24 h after plating with fluorescein-labelled tubulin and further

incubated for 10-24 h. A small area of the axon was illuminated with a halogen lamp (12 V, 50 W) and fluorescence observations were made with a Nikon X100 fluor objective lens on an Olympus IMT-2 inverted microscope equipped with an SIT camera (Hamamatsu Photonics, C2400-08) and a digital image processor (Hamamatsu Photonics, ARGUS-100). Video frames were summed, averaged for 1/2 s, and background fluorescence subtracted. Fluorescein photobleaching was performed using an argon ion laser at 3-5 mW for 8 ms. The photobleaching apparatus was designed and assembled according to the method of Hamaguchi *et al.*³⁰. Anti-tubulin staining of the injected cells was as previously described¹¹.

FIG. 2 Elongation of a fluorescent neurite distal to the bleached zone. DRG neurons were microinjected with fluorescein-labelled tubulin 2–4 h after plating, incubated for 4–6 h and then observed (a). A region of the axon just proximal to the growth cone was bleached with a laser beam (arrow in b) and observed 70 min (c) and 120 min (d) later. The growth cone was still active after photobleaching but the bleached zone remained in the same position (arrows in c and d), resulting in a significant elongation of the fluorescent neurite distal to the bleached zone. Bar, 10 μm .



the same axonal position for ~ 1 h after photobleaching (Fig. 1a, c). This was not because of the breakdown of microtubules, as anti-tubulin staining revealed that bleached microtubules remained intact regardless of the time that had elapsed since they were bleached (Fig. 1d). Tubulin molecules are transported down the axon at a rate of 0.25–1 μm per day⁶. If tubulin molecules are transported as polymers, bleached microtubules should move >10 μm within 1 h. But we did not observe movement or spreading of the bleached zones, indicating that microtubules do not move down the axon.

To determine whether a translocation of microtubules occurs in actively growing neurites, we bleached a region of the axon just proximal to the advancing growth cone (Fig. 2). After photobleaching, the growth cone was still active and continued to advance. By contrast, the bleached zone was stationary, resulting in a significant elongation of the fluorescent neurite distal to the bleached zone. This result indicates that the translocation of microtubules does not occur during the process of neurite elongation, whereas net transport of tubulin molecules to the neurite tip is necessary.

Instead of moving down the axon, the bleached zones remained in the same position and recovered fluorescence gradually (Fig. 1e–h). To confirm this, we quantified the turnover of tubulin by measuring fluorescence photobleaching recovery (FPR)¹⁷ (Fig. 4a). We first characterized the recovery within 60 s using the method of optimal superposition of log-log plots of experimental data and theoretical recovery curves^{17,18}. The results indicate that the first component of the recovery can be explained by the diffusion of free subunits (Fig. 4b), and the diffusion coefficient (D) was calculated to be $4.5(\pm 0.74) \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$, which is in the range of previously reported values of D for macromolecules^{19,20}. Although the theoretical curve indicates that for the first component the time for half of the fluorescence to recover (the recovery half-time) would be ~ 20 s (Fig. 4b), the FPR data show that the fluorescence reached only 10–15% of the initial value within 60 s (Fig. 4a). This result indicates that the first higher-mobility component corresponds to only 13–20% of the total fluorescence recovery and there are at least two components in the recovery process. To characterize the recovery of the second component, we quantified the relative recovery of fluorescence in the bleached zone for each stored image of video microscopy (Fig. 4c). The second component recovered more slowly over the course of minutes and could represent the recovery limited by chemical exchange between tubulin subunits and polymers. The observed spatio-temporal pattern of fluorescence recovery indicates that axonal microtubules are stationary but dynamic. These results are fully consistent with our previous study¹¹ and recent work by Lim *et al.*¹⁴.

DRG neurons fully incorporated fluorescein-labelled actin 3–12 h after microinjection (Fig. 3a, f). We measured FPR to determine whether the observed pattern of fluorescence mainly reflects the distribution of filamentous actin or the distribution of monomeric actin (Fig. 4a). As for tubulin molecules, there were at least two components in the recovery process. The recovery of the higher-mobility component can be explained by

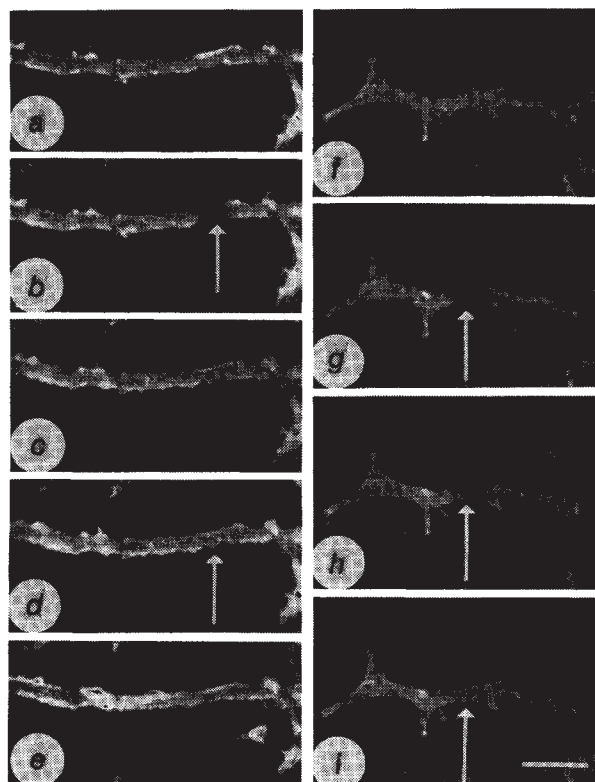
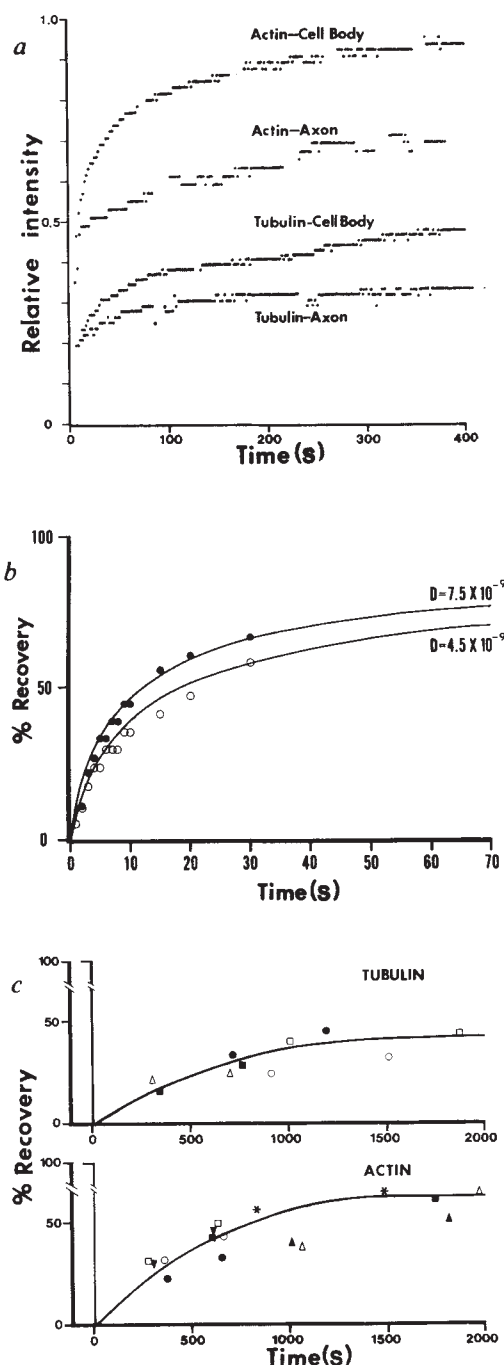


FIG. 3 Fluorescence recovery after photobleaching of fluorescein-labelled actin in the axons. a–e, Four successive frames of video microscopy showing an axon before (a), immediately after (b), 17 min after (c), and 30 min after (d) photobleaching. The bleached zone (arrow in b) did not move down the axon but recovered its fluorescence significantly 30 min after photobleaching (arrow in d). The same axon was fixed 50 min after photobleaching and the distribution of actin filaments was analysed by staining with rhodamine-phalloidin (e). There was no detectable change in the distribution of filamentous actin in the bleached zone. f–i, Recovery of fluorescence in the bleached zone. The fluorescent axon was observed before (f), immediately after (g), 5 min after (h), and 10 min after (i) photobleaching. The bleached zone (arrows in g–i) recovered its fluorescence gradually. Left side of figures is distal to the cell body. Bar, 10 μm .

METHODS. Actin was labelled with 5(6)-carboxy-fluorescein succinimidyl ester²⁸. Rhodamine-phalloidin staining of DRG neurons was as previously described²⁶.



diffusion (Fig. 4b), and the value of D for actin was estimated to be $7.5 (\pm 1.2) \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$ (recovery half-time, ~ 15 s). The higher-mobility component was prominent in the cell body ($>50\%$ of the fluorescence recovery), but was a minor component in the axon, indicating that the amount of freely diffusible molecules is small in the axon.

To further characterize the mechanism of actin turnover, the position of the bleached zone in the axon was analysed. If actin filaments are static polymers travelling down the axon at the rate of 2–4 mm per day⁶, bleached actin filaments should move $>10 \mu\text{m}$ within 10 min. But we did not observe movement or spreading of the bleached zone 10 min after bleaching (Fig. 3g, i), indicating that actin filaments are not static polymers transported down the axon. Although actin filaments did not move down the axon, the data of the FPR measurements indicated that they underwent a slow turnover along the length of

FIG. 4 Quantitative analysis of fluorescence recovery within bleached regions. *a*, FPR measurements of fluorescein-labelled tubulin and actin. Fluorescence recovery of actin molecules in the cell body was prominent in the early phase (<100 s), indicating a large fraction of highly mobile actin molecules in the cell body. In the late phase of the recovery (>100 s), fluorescence of actin in the axon recovered more rapidly than that of tubulin. *b*, Comparison of the theoretical curves of fluorescence recovery with the FPR data. The optimal superposition of log-log plots of experimental data and theoretical recovery curves has revealed that the early phase of the recovery can be explained by diffusion of free subunits, and the value of D was calculated to be $4.5 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$ for tubulin and $7.5 \times 10^{-9} \text{ cm}^2 \text{ s}^{-1}$ for actin. The theoretical curves of fluorescence recovery due to diffusion were plotted together with the typical FPR data for tubulin ($\circ$) and actin ($\bullet$). The plots indicate that the early fluorescence recovery is likely to be driven by diffusion of free molecules. *c*, The late phase of fluorescence recovery within the bleached regions of the axons. The relative recovery of fluorescence in the bleached zone for each stored image of video microscopy was quantified and the percentage recovery was plotted against time after bleaching. Each symbol represents data from an individual bleached zone. These plots indicate that the turnover of actin is faster than that of tubulin during the late phase of fluorescence recovery.

METHODS. For FPR measurements, a small region of injected cells was bleached by a laser beam at 3–5 mW for 8 ms and the bleaching beam was attenuated 10,000 times for the measurement of fluorescence recovery. The fluorescence intensity was measured every 2 s and the data were stored in a digital image processor. The theoretical fluorescence recovery curves for diffusion were determined for an idealized condition where one-dimensional diffusion was monitored by a laser beam of uniform intensity, the width of which was $6 \mu\text{m}$ along the axon. A series solution for the percentage recovery $F(t)$ is given by

$$F(t) = \frac{4L}{\pi^2} \sum_{n=1}^{\infty} (\sin(n\pi w/L)/n)^2 (1 - e^{-(n\pi/L)^2 Dt})$$

where L is the length of the system, w is the length of the bleached zone and D is the diffusion coefficient. The method of optimal superposition of log-log plots has been described previously^{17,18}.

the axon. As for microtubules, this gradual turnover is likely to be driven by the assembly–disassembly of actin filaments.

If tubulin and actin do not move in the form of polymers, then soluble free subunits or small oligomers can be expected to be mainly transported down the axon^{7–11}. In such a case, soluble subunits generated by the local disassembly of stationary polymers would be transported for a certain distance and then added to the pre-existing polymers and become stationary again. This model indicates that the rate of exchange between soluble and polymeric components would be one of the main factors determining the average rate of transport.

Because tubulin and actin are transported at different rates⁶, we determined whether there is a difference in the rates of assembly–disassembly for these two proteins. The FPR measurements (Fig. 4a) show that the recovery of the lower-mobility component of actin is faster than that of tubulin. Quantification of the stored images of video microscopy also reveal that the slow recovery of tubulin and actin differ (Fig. 4c). Turnover of actin was faster than that of tubulin in the axon, and the recovery half-time was roughly estimated to be 45–90 min for tubulin and 15–30 min for actin. It is possible, therefore, that the distinct velocities of slow axonal transport are generated by the difference in the exchange rates of these two cytoskeletal systems. The presence of a tubulin wave moving at velocities comparable to actin has been reported in certain neural systems²¹. This faster wave contained a larger fraction of cold-soluble more-dynamic tubulin²², further indicating the relation between the rate of transport and the rate of assembly–disassembly.

Recent studies on the dynamics of the cytoskeleton have revealed that cytoskeletal polymers continue to exchange their subunits within living cells^{23–27}. Although the axoplasm is a highly specialized region of the cytoplasm, the results from the experiments described here, together with several previous

studies⁷⁻¹¹, indicate that the dynamics of the cytoskeleton in neurons and other non-neuronal cells share common basic properties. □

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- Mitchison, T. & Kirschner, M. *Neuron* **1**, 761-772 (1988).
- Hirokawa, N. *J. Cell Biol.* **94**, 129-142 (1982).
- Hirokawa, N., Glicksman, M. A. & Willard, M. B. *J. Cell Biol.* **98**, 1523-1536 (1984).
- Hirokawa, N., Bloom, G. S. & Vallee, R. B. *J. Cell Biol.* **101**, 227-239 (1985).
- Willard, M., Cowan, W. M. & Vagelos, P. R. *Proc. natn. Acad. Sci. U.S.A.* **71**, 2183-2187 (1974).
- Black, M. M. & Lasek, R. J. *J. Cell Biol.* **86**, 616-623 (1980).
- Bamburg, J. R., Bray, D. & Chapman, K. *Nature* **321**, 788-790 (1986).
- Nixon, R. A. & Logvinenko, K. B. *J. Cell Biol.* **102**, 647-659 (1986).
- Weisenberg, R. C. *et al. Science* **238**, 1119-1122 (1987).
- Kosik, K. S. & Finch, E. A. *J. Neurosci.* **7**, 3142-3153 (1987).
- Okabe, S. & Hirokawa, N. *J. Cell Biol.* **107**, 651-664 (1988).
- Okabe, S. & Hirokawa, N. *Proc. natn. Acad. Sci. U.S.A.* **86**, 4127-4131 (1989).
- Hollenbeck, P. J. *J. Cell Biol.* **108**, 223-227 (1989).
- Lim, S.-S., Sammak, P. J. & Borisy, G. G. *J. Cell Biol.* **109**, 253-263 (1989).
- Vigers, G. P. A., Coue, M. & McIntosh, J. R. *J. Cell Biol.* **107**, 1011-1024 (1988).
- Keith, C. H. *Science* **235**, 337-339 (1987).
- Axelrod, D., Koppel, D. E., Schlessinger, J., Elson, E. L. & Webb, W. W. *Biophys. J.* **16**, 1055-1069 (1976).
- Wolf, D. E. *Meth. Cell Biol.* **30**, 271-306 (1989).
- Kreis, T. E., Geiger, B. & Schlessinger, J. *Cell* **29**, 835-845 (1982).
- Salmon, E. D., Saxton, W. M., Leslie, R. J., Karow, M. L. & McIntosh, J. R. *J. Cell Biol.* **99**, 2157-2164 (1984).
- Oblinger, M. M., Brady, S. T., McQuarrie, I. G. & Lasek, R. J. *J. Neurosci.* **7**, 453-462 (1987).
- Tashiro, T. & Komiya, Y. *J. Neurosci.* **9**, 760-768 (1989).
- Sammak, P. J. & Borisy, G. G. *Nature* **332**, 724-726 (1988).
- Schulze, E. & Kirschner, M. *Nature* **334**, 356-359 (1988).
- Wang, Y. L. *J. Cell Biol.* **101**, 597-602 (1985).
- Okabe, S. & Hirokawa, N. *J. Cell Biol.* **109**, 1581-1595 (1989).
- Vikstrom, K. L., Borisy, G. G. & Goldman, R. D. *Proc. natn. Acad. Sci. U.S.A.* **86**, 549-553 (1989).
- Kellog, D. R., Mitchison, T. J. & Alberts, B. M. *Development* **103**, 675-686 (1988).
- Goldenberg, S. S. & De Boni, U. *J. Neurobiol.* **14**, 195-206 (1983).
- Hamaguchi, Y., Toriyama, M., Sakai, H. & Hiramoto, Y. *Cell Struct. Funct.* **12**, 43-52 (1987).

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Uncoupling of the synthesis of edited and unedited COIII RNA in *Trypanosoma brucei*

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RNA EDITING, a novel and unexpected type of information processing, was first demonstrated in the kinetoplasts of certain protozoans¹⁻¹². It is a remarkable phenomenon: certain species of messenger RNA have nucleotide sequences that differ greatly from the sequences of the genes from which they are presumably transcribed. The differences are usually due to addition of uridylyte residues, but occasionally also to their deletion. The most spectacular case of editing known occurs in the mRNA of the mitochondrial gene for subunit III of cytochrome oxidase (COIII) in *Trypanosoma brucei*. This mRNA is twice the length of its gene owing to the addition of several hundred uridylyte residues and a few deletions, spread over the entire length of the RNA molecule¹³. Whereas unedited RNA molecules, the nucleotide sequences of which correspond to the genomic sequence, have been isolated¹³⁻¹⁶, no DNA template corresponding to the edited RNA sequence has been detected in either the mitochondrial or nuclear genome. It was suggested, therefore, that unedited mRNAs are transcribed from mitochondrial DNA and then edited post-transcriptionally by endonucleolytic cleavage of the primary transcript at specific sites, followed by insertion or deletion of uridylyte residues and religation¹³. We have now examined the general nature of the RNA editing process to determine whether it involves the insertion of nucleotide residues into pre-existing molecules or the continuous

de novo synthesis of edited mRNA. The results of our experiments rule out an insertion mechanism and strongly indicate that edited mRNA is synthesized as a unit.

Our approach to examining the general nature of RNA editing involved uncoupling the synthesis of unedited RNA and the editing process by the use of specific inhibitors. We aimed to label cells with nucleotide precursors while the synthesis of unedited RNA was selectively inhibited, to isolate edited RNA species, and to measure the relative specific activities of the four nucleotides. If editing occurs by insertion of uridines into pre-existing unedited RNA, only uridines would be labelled. But if editing involves *de novo* RNA synthesis, that is, if edited RNA is synthesized as a unit, all four nucleotides would be labelled.

To define conditions for selectively inhibiting the synthesis of unedited RNA for subunit III of cytochrome oxidase we used various inhibitors of nucleic acid synthesis and various experimental conditions such as heat shock (Table 1, Fig. 1). Rifampicin, reported to inhibit mitochondrial RNA synthesis, had little effect on the synthesis of either edited or unedited RNA. Ethidium bromide inhibited synthesis of both edited and unedited RNA. Heat shock enhanced synthesis of unedited RNA, but had little effect on the synthesis of edited RNA. But we obtained a most striking result with actinomycin D, which completely inhibited the synthesis of unedited COIII RNA, but had almost no effect on the synthesis of edited COIII RNA. We saw this effect both when measuring the incorporation of radioactivity into edited and unedited COIII RNA (Table 1) and when measuring the steady-state levels of edited and unedited COIII RNA in cells treated with actinomycin D for different lengths of time (Fig. 1). Whereas the levels of unedited COIII RNA declined rapidly in the presence of the inhibitor, the levels of edited RNA remained relatively constant. Similar inhibition of synthesis of unedited but not of edited RNA by actinomycin D is obtained for cytochrome *b* RNA and MURF-2 RNA of *T. brucei* (our unpublished observations). The most straightforward interpretation of these results is that actinomycin D selectively inhibits synthesis of unedited but not of edited COIII RNA. In this case, the continued synthesis of edited RNA in the absence of synthesis of unedited RNA implies that unedited COIII RNA

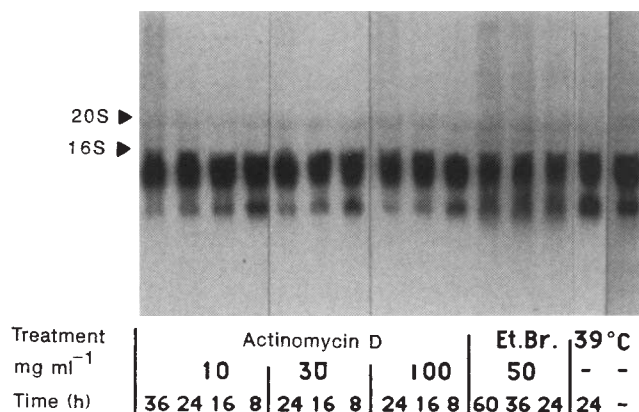
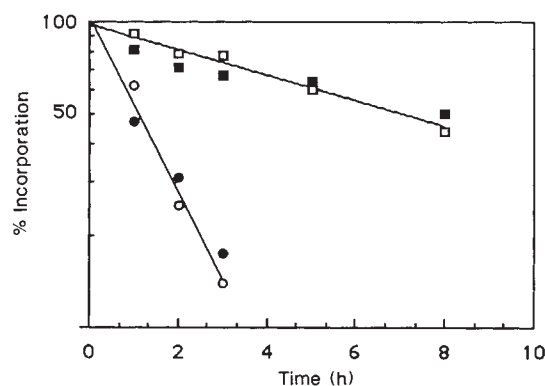


FIG. 1 Effect of actinomycin D, ethidium bromide and elevated temperature (39 °C) on levels of unedited and edited COIII RNA. Cells were treated as indicated. Abbreviations: Et.Br., ethidium bromide; C, control. RNA was isolated and northern blots containing total cytoplasmic RNA were prepared¹⁸ and hybridized with a mixture of end-labelled oligonucleotides¹⁹ (Probes 1 and 2; Table 1) as described elsewhere (V.V., B.S. and S.R., manuscript submitted). Probe 1 specifically detects unedited COIII RNA (lower band), whereas probe 2 hybridizes specifically to edited COIII RNA (upper band). The arrows show the position of prominent 16S and 20S RNA components from trypanosomes, present in all samples, which serve as convenient internal standards.



is not a precursor of its edited counterpart. Another, although more complex, interpretation of these results is that actinomycin D does not inhibit the synthesis of unedited COIII RNA, but rather enhances its instability by accelerating its conversion into edited RNA. But this alternative is ruled out by the observation that actinomycin D had no effect on the rate of turnover of either unedited or edited RNA (Fig. 2). To measure the rates of turnover of edited and unedited COIII RNA, we labelled cells with a pulse of tritiated uridine, followed by a chase with unlabelled uridine and cytidine. We isolated cytoplasmic RNA after various chase periods and assayed it by hybridization with an excess of an immobilized probe specific for the 3' end of unedited RNA or for the 5' portion of edited COIII RNA. The

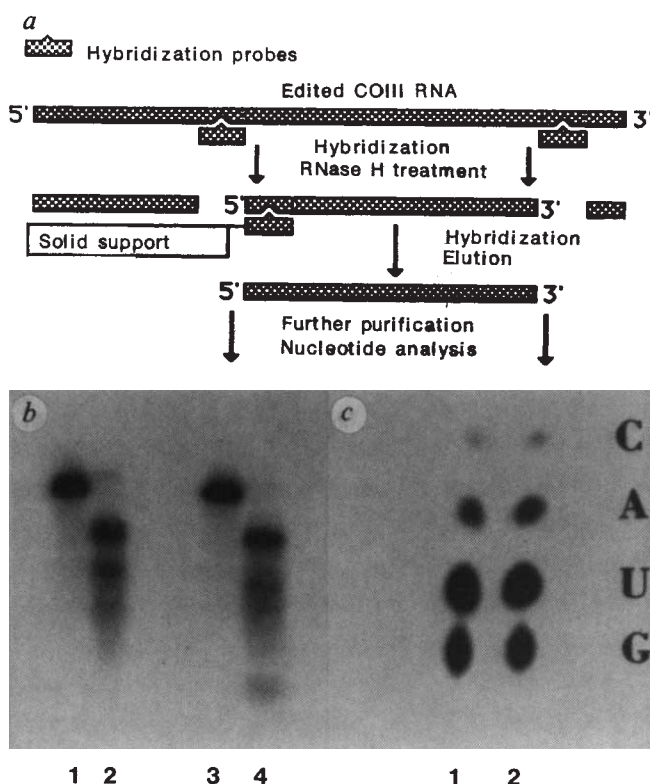
FIG. 2 Effect of actinomycin D on rates of turnover of unedited and edited COIII RNA. Cells (3×10^5 per ml) were labelled with [3 H]uridine ($100 \mu\text{Ci ml}^{-1}$) for 2 h, washed twice with growth medium, and resuspended in fresh growth medium with (■, ●) or without (□, ○) actinomycin D supplemented with 10 mM unlabelled uridine and 5 mM cytidine. Cytoplasmic RNA was hybridized to an excess of immobilized oligonucleotide 1, complementary to a 3'-end portion of unedited COIII RNA (□, ■) or oligonucleotide 2, complementary to a 5' portion of edited COIII RNA (○, ●) (see Table 1). Labelling, isolation of RNA and hybridization were as described elsewhere (V.V., B.S. and S.R., manuscript in preparation).

half-lives of unedited and edited RNA were 7 h and 75 min, respectively, and were unaffected by the presence of actinomycin D at concentrations that strongly suppressed labelling of unedited RNA. The effect of actinomycin D on the labelling and steady-state levels of unedited RNA is therefore due to the inhibition of the synthesis of unedited RNA.

If we assume that the two probes of Fig. 1, which have nearly the same specific radioactivity, have similar hybridization efficiencies, then our data indicate a lack of precursor-product relationship between unedited and edited RNA. Indeed, if there is such a precursor-product relationship, the steady-state level of edited COIII RNA should be lower than that of unedited RNA, because the production of edited COIII RNA could at

FIG. 3 Characterization and nucleotide composition of purified labelled fragment of edited COIII RNA from cells treated and untreated with actinomycin D. a, Schematic illustration of the strategy for purification of a specific fragment of edited COIII RNA. b, RNase H restriction assay for homogeneity of RNA preparation. c, Nucleotide analysis.

METHODS. To isolate a fully edited specific fragment of COIII RNA, cells (2×10^6) were labelled for 4 h with $^{32}\text{P}_i$ in the presence or absence of actinomycin D ($100 \mu\text{g ml}^{-1}$, added 12 h before labelling), and cytoplasmic RNA hybridized to a mixture of an excess of two oligonucleotides, 5'-CTCACACACAAAAATAAACATAAACCAATAATACAACAAAAACGCC-3' and 5'-CAAACTACCAATACAAATTAATACTAACAACAACTAA-3', complementary to the 5' and 3' portions, respectively, of the sequenced part of edited COIII RNA. Hybridization was followed by phenol-chloroform extractions and precipitation with ethanol. Precipitated material was resuspended in 100 μl of 20 mM HEPES buffer, pH 8.0, 70 mM NaCl, 10 mM MgCl_2 , and 1 mM DTT, and incubated with 5 U RNase H for 1 h at 37 °C. An equal volume of 0.2 M Tris-HCl buffer, pH 7.5, 0.24 M NaCl, 25 mM EDTA, 2% SDS, and 400 mg ml^{-1} proteinase K was then added and the incubation continued for 1 h. After phenol-chloroform extractions and precipitation, the RNA resistant to RNase H was hybridized to immobilized oligonucleotide 5'-CCATAATCACATAATAACAACAAAACATATAAGGTAAACAAAAACGAAAG-CAAA-3' complementary to the 5' end of RNA fragment between the sites of cutting by RNase H, washed at 37 °C with 5 $\times$ SSPE (1 $\times$ SSPE: 90 mM NaCl, 10 mM NaH_2PO_4 , 1 mM EDTA, pH 7.4), 0.2% SDS; 2 $\times$ SSPE, 0.2% SDS, 0.5 $\times$ SSPE, 0.2% SDS, and eluted with 10 mM Tris-HCl buffer, pH 7.5, 1 mM EDTA at 68 °C. The eluted material was precipitated in the presence of 20 μg yeast RNA as carrier and electrophoresed on a 1.8% gel. The resulting 530-nucleotide fragment was visualized by autoradiography and electroeluted from the gel in 50 mM Tris-boric acid buffer, 10 mM EDTA, pH 8.0. To test the homogeneity of the resulting RNA preparations, a sample was hybridized in solution to an excess of oligonucleotide 5'-CGCATAAAACAAAT-AAACAACAACAAAACACC-3' complementary to a central portion of an expected RNA fragment, treated with RNase H and analysed by agarose gel electrophoresis as described above. To analyse the nucleotide composition of the isolated RNA, the RNA preparations were dissolved in 8 μl of 30 mM sodium acetate, pH 5.3, and 0.3 mM ZnSO_4 , and incubated with 2 U P1 nuclease for 1 h at 37 °C. The mixtures were then heated for 10 min at 70 °C, adjusted to 0.2 M Tris-HCl buffer, pH 8.9, and incubated with 4 μg *Crotalus durissus* phosphodiesterase for 2 h at 25 °C. The resulting labelled material was analysed by TLC as previously described²⁰, except that the plates were first washed with 0.2 M LiOH, adjusted to pH 3.5 with formic acid, and a second development was carried out with the same solvent. b, Lane 1—RNA fragment (530 nucleotides) from actinomycin D-treated cells



electroeluted from the gel; lane 2—same RNA as in lane 1, after RNase H restriction assay; lane 3—RNA fragment (530 nucleotides) from untreated cells electroeluted from the gel; lane 4—same RNA as in lane 3, after RNase H restriction assay. The size of two predominant fragments in lanes 2 and 4 was as expected (336 nucleotides and 162 nucleotides, respectively). c, Lane 1—nucleotide analysis of RNA actinomycin D-treated cells; lane 2—nucleotide analysis of RNA from untreated cells.

TABLE 1 Effects of heat shock and inhibitors of nucleic acid synthesis

Treatment		³² P Incorporated (c.p.m. in 3 h) into:	
		Unedited RNA	Edited RNA
None		1,673	7,024
Actinomycin D	10 µg ml ⁻¹	242	7,893
	30 µg ml ⁻¹	82	7,611
	100 µg ml ⁻¹	41	6,881
Rifampicin	15 µg ml ⁻¹	1,421	7,264
	50 µg ml ⁻¹	1,811	6,785
	150 µg ml ⁻¹	1,521	6,312
Ethidium bromide	30 µg ml ⁻¹	624	2,670
	50 µg ml ⁻¹	510	2,121
	100 µg ml ⁻¹	398	1,458
39 °C		4,122	6,091

Cells (2.2×10^8) of *T. brucei* were labelled with ³²P_i for 3 h under conditions in which incorporation of radioactivity was linear for at least 4 h. Inhibitors were added 1 h before labelling. Cytoplasmic RNA was isolated and hybridized to an excess of immobilized oligonucleotides as described elsewhere (V.V., B.S. and S.R., manuscript submitted). The following oligonucleotides were used: 5'-ACCTCTTCATCCAAC-TAAACCCTTTCCCTCTCTCTCTCGCTTCTCTAAATCCTGTC-3'⁽¹⁾ complementary to the 3' portion of unedited COIII RNA; and 5'-GGTAAACAAAAAC-GAAAGCAAACACACAAAAATAAACATAAACCAAAT-3'⁽²⁾ complementary to the 5' portion of edited COIII RNA¹³.

most correspond to the decay of the unedited RNA, but its degradation would be faster. However, the steady-state level of the edited COIII RNA is actually higher.

The ability to inhibit the synthesis of unedited but not of edited RNA allowed us to investigate further the relationship between unedited and edited COIII RNA by the nucleotide-labelling experiment proposed above. We labelled cells with ³²P_i in the presence (or absence) of actinomycin D, isolated the labelled cytoplasmic RNA, and hybridized it in solution to an excess of two oligodeoxynucleotides—one complementary to the 5'-terminal portion of edited COIII RNA and the other complementary to its 3' end (Fig. 3). We treated the hybrid with RNase H, and purified the residual RNA of interest by hybridization and elution from an immobilized oligodeoxynucleotide complementary to the 5' end of the edited COIII RNA segment between the two sites of RNase H digestion. This procedure produces a large fragment (530 nucleotides) of edited COIII RNA that is fully edited (data not shown), and has defined ends and a known nucleotide sequence¹³. The RNA that we obtained was further purified by electrophoresis, visualized by autoradiography and electroeluted from the gel. To test the homogeneity of the resulting RNA preparation, we hybridized a sample to an oligodeoxynucleotide complementary to a central portion of the edited COIII RNA, treated it with RNase H, and analysed it by gel electrophoresis. This 'restriction' assay converted the material into two new fragments of the expected size (336 nucleotides and 162 nucleotides), confirming the homogeneity of the purified RNA (Fig. 3b). We determined the nucleotide composition of the labelled purified RNA by complete sequential digestion with P1 nuclease and snake venom phosphodiesterase, followed by TLC of the resulting 5' nucleotides (Fig. 3c). The edited COIII RNA fragment obtained from RNA labelled in the presence of actinomycin D yielded four labelled nucleoside monophosphates with a C:A:U:G ratio of 3:11:58:28 (average of two separate experiments), roughly corresponding to the relative amounts of these nucleoside monophosphates in this RNA fragment (C:A:U:G ratio of 3:14:61:22)¹³. We obtained similar results in the absence of actinomycin D. That the four nucleotides of edited RNA were uniformly labelled in the presence of actinomycin D, under

conditions when the synthesis of unedited RNA was inhibited, strongly indicates that edited COIII RNA is synthesized as a unit. If synthesis of edited RNA occurs by the insertion of uridylylate residues in pre-existing unedited RNA, only UMP should have been labelled in the presence of actinomycin A. (We note that this conclusion applies to the bulk of edited RNA, but that the methods we used were not sensitive enough to detect a small fraction of edited RNA that might have been produced by an insertional process.)

We conclude that there is no direct precursor-product relationship between unedited and edited COIII RNA, and that the bulk of edited RNA is synthesized *de novo* as a unit. The synthesis of edited RNA must therefore involve a transcriptional process, and the great challenge at this stage is to discover the template from which edited RNA is transcribed. □

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1. Eisen, H. *Cell* **53**, 331–332 (1988).
2. Maizels, N. & Weiner, A. *Nature* **334**, 469–470 (1988).
3. Lamond, A. *Trends Biol. Sci.* **13**, 283–284 (1988).
4. Simpson, L. & Shaw, J. *Cell* **57**, 355–366 (1989).
5. Benne, R. *Biochim. biophys. Acta* **1007**, 131–139 (1989).
6. Stuart, R. *Parasitol. Today* **5**, 5–8 (1989).
7. Benne, R. *et al. Cell* **46**, 819–826 (1986).
8. Feagin, J. E., Jasmer, D. P. & Stuart, K. *Cell* **49**, 337–345 (1987).
9. Feagin, J. E. & Stuart, K. *Molec. cell. Biol.* **8**, 1259–1265 (1988).
10. Feagin, J. E., Shaw, J. M., Simpson, L. & Stuart, K. *Proc. natn. Acad. Sci. U.S.A.* **85**, 539–543 (1988).
11. Shaw, J. M., Feagin, J. E., Stuart, K. & Simpson, L. *Cell* **53**, 401–411 (1988).
12. Van der Spek, H. *et al. EMBO J.* **7**, 2509–2514 (1988).
13. Feagin, J. E., Abraham, J. M. & Stuart, K. *Cell* **53**, 413–422 (1988).
14. Benne, R., De Vries, B. F., Van den Burg, J. & Klaver, B. *Nucleic Acids Res.* **11**, 6925–6941 (1983).
15. Hensgens, L. A. M. *et al. Nucleic Acids Res.* **12**, 7327–7344 (1984).
16. Feagin, J. E., Jasmer, D. P. & Stuart, K. *Nucleic Acids Res.* **13**, 4577–4596 (1985).
17. Abraham, J. M., Feagin, J. E. & Stuart, K. *Cell* **55**, 267–272 (1988).
18. Volloch, V., Schweitzer, B. & Rits, S. *Expl. Cell. Res.* **173**, 38–48 (1987).
19. Maniatis, T., Fritsch, E. F. & Sambrook, J. *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, New York, 1982).
20. Voickaert, G. & Fiers, W. *Analyt. Biochem.* **83**, 222–227 (1977).

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RNA bulges and the helical periodicity of double-stranded RNA

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RNA MOLECULES typically exhibit extensive secondary structure, including double-stranded duplex, hairpins, internal loops, bulged bases and pseudoknotted^{1,2} structures (reviewed in refs 3 and 4). This is intimately connected with biological function, including splicing reactions^{5,6} and ribozyme activity^{7,8}. The formation of RNA–DNA hybrids is important in the transcription of DNA, reverse transcription of viral RNA, and DNA replication. Bulged bases in RNA helices are potentially significant in RNA folding and in providing sites for specific protein–RNA interactions, as illustrated by TFIIIA of *Xenopus*⁹ and the coat protein of phage R17 (ref. 10). Most information about the structure of RNA derives from fibre diffraction^{11,12} or crystallography of natural molecules, notably transfer RNA^{13–17}, but until recently there have been few systematic studies of RNA structure using designed sequences^{18–22}. We have used gel electrophoresis to investigate the properties of bulged bases in both RNA and RNA–DNA duplexes in solution. As in DNA helices^{23–25}, bulges introduce pronounced kinks into RNA and into RNA–DNA helices,

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depending on the number and types of bases in the bulge and its position in the fragment. By varying the spacing between two bulge-induced kinks, we have measured the periodicity of RNA and RNA-DNA helices in solution.

Bulges (unopposed bases on one strand of a duplex) introduce a pronounced bend or kink into duplex DNA molecules²³⁻²⁵, which is consistent with NMR studies of single-base bulges²⁶. This results in a markedly decreased mobility on gel electrophoresis; further analysis by this technique reveals that the change in the trajectory of the helix axis is centred on the bulge itself. This indicates that the bulge probably has precise molecular structure which might render it a suitable target for recognition by proteins. It seemed to us that this was more likely to be important in RNA molecules, so we investigated whether a similar structure could form in RNA duplexes and in RNA-DNA hybrid duplexes. We constructed 40-base pair duplexes, together with duplexes containing a centrally positioned, five-base adenine bulge, from RNA species generated by T7 RNA polymerase transcription of synthetic oligodeoxynucleotides. RNA-DNA duplexes were constructed by hybridization of synthetic DNA strands to RNA transcripts, such that the bulges

were present on the RNA strand. The gel electrophoretic mobilities of both the perfect duplex and bulged species for DNA, RNA and RNA-DNA are shown in Fig. 1. The three different non-bulged helices exhibited markedly different gel mobilities, in the order DNA > RNA-DNA > RNA, which is consistent with different helical geometries for the three species. The intermediate migration of the hybrid duplex is interesting, as such molecules have generally been considered to adopt an A-type helix, similar to RNA, in which the RNA and DNA strands have C3'-*endo* and C2'-*endo* ribose conformations respectively^{12,27}; these two species might therefore be expected to co-migrate on the gel. The intermediate mobility of the RNA-DNA duplex is consistent with some kind of hybrid geometry. In all three types of duplex, the introduction of a five-base adenine bulge produced a retardation effect on gels; this was more pronounced in the DNA than in the RNA helix, with the hybrid duplex displaying an intermediate retardation. This indicates that all three species may be kinked by the formation of a bulge, but that the magnitude of the kink is greater in DNA than it is in an RNA helix for the same sequences and size of bulge.

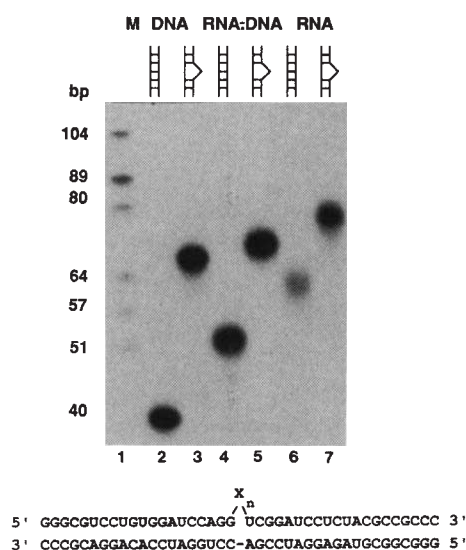


FIG. 1 Retardation on polyacrylamide gel electrophoresis of double-stranded DNA, RNA-DNA and double-stranded RNA as a result of bulge formation. ³²P-labelled 40-bp duplexes of each species having identical sequences were electrophoresed in 15% polyacrylamide, alongside equivalent duplexes containing a bulge of five adenine bases. The sequence of the double-stranded RNA fragment is shown; X_n indicates A₅ in this experiment. The hybrid molecule was constructed so that the bulge was located on the RNA strand. Lane 1 (M), DNA restriction fragments as size markers (pAT153 digested with *Hae*III) with lengths indicated on the left; lane 2, DNA duplex; lane 3, DNA duplex with bulge; lane 4, RNA-DNA hybrid duplex; lane 5, RNA-DNA duplex with bulge; lane 6, double-stranded RNA duplex; lane 7, RNA duplex with bulge.

METHODS. Oligodeoxyribonucleotides were synthesized using β -cyanoethylphosphoramidite chemistry^{31,32} implemented on an Applied Biosystems 381 synthesizer. Oligoribonucleotides were synthesized by transcription from synthetic DNA templates using T7 RNA polymerase³³. Homogeneously radioactively labelled RNA species were prepared by transcription in 40 mM Tris-HCl (pH 7.5), 6 mM MgCl₂, 2 mM spermidine and 0.5 mM each of ATP, GTP, CTP and UTP together with [α -³²P] CTP for 3 h at 37 °C, and then purified by 10 or 12% polyacrylamide gel electrophoresis. Complementary DNA and/or RNA oligonucleotides were mixed in equimolar quantities (2 nM of each species) and hybridized in 0.45 M sodium citrate, 0.045 M NaCl for 15 min at 65 °C, followed by slow cooling to room temperature. Nucleic acid duplexes were electrophoresed in 15 or 20% polyacrylamide gels in 90 mM Tris-borate (pH 8.3), 50 mM NaCl at 15 $\pm$ 0.1 °C for 16 h. Bands were visualized by autoradiography.

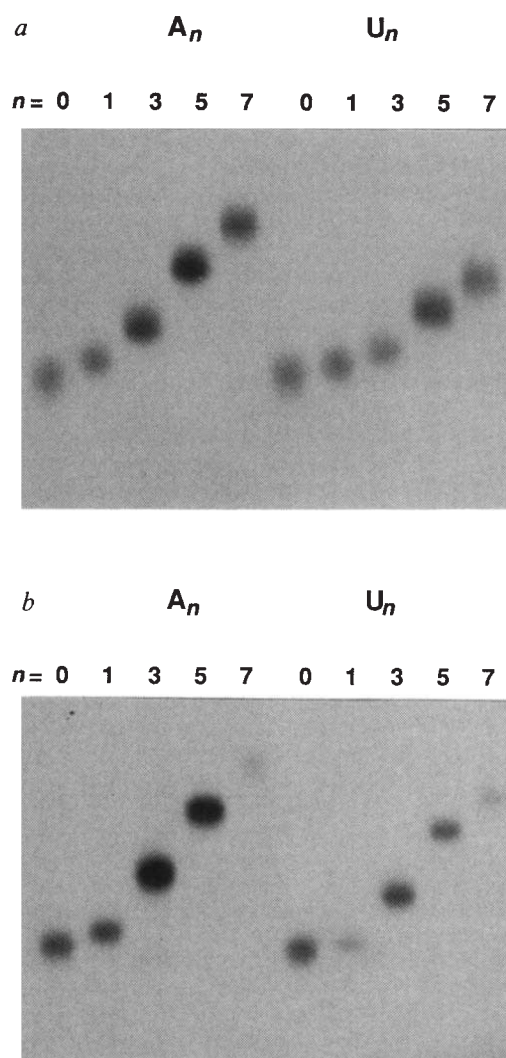
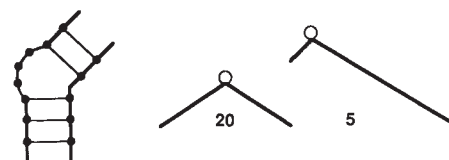
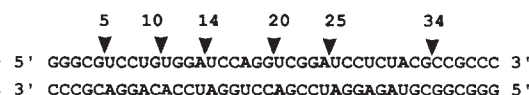
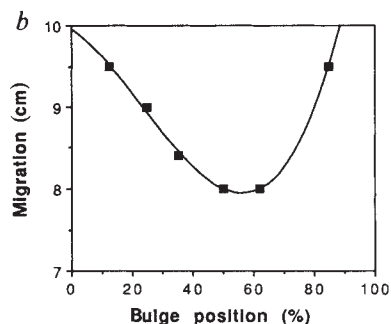


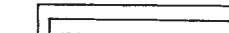
FIG. 2 Electrophoretic mobility of RNA fragments depends on the number (*n*) and type of bulged bases. 40-bp RNA (*a*) and RNA-DNA (*b*) duplexes were constructed with central bulges containing 0, 1, 3, 5 or 7 bases, which were either adenine or uracil, and electrophoresed in 15% polyacrylamide gels. The autoradiographs of these gels are shown. The sequences of the molecules are identical to those in Fig. 1.

retardation, and its position-dependence, indicates that the bulge causes a sharp change in trajectory of the RNA helical axis, thus kinking the RNA molecule as indicated (*b*). Such a kink will generate molecules with markedly different shapes, and therefore electrophoretic mobilities, depending on its location in the molecule as shown.

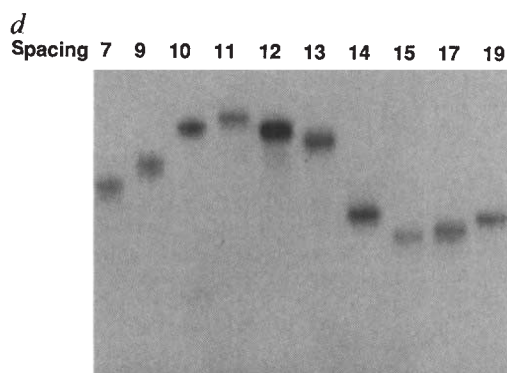
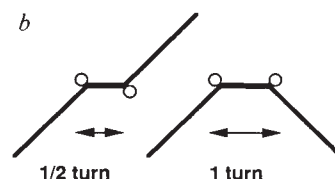


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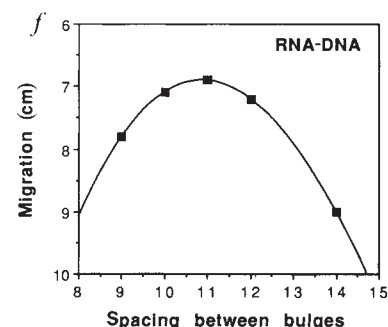
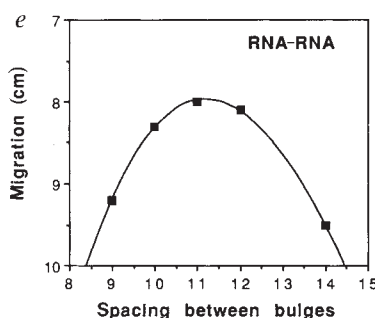
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5' GGGCGUCCUGUGGAUCCAGGUGGUCACAGCUUUGUCUGUAAUCCGAUCCUCUACGCCGCCCC 3'
3' CCCGCAGGACACCUAGGUCCACCAGUGUGCGAACAGACAUUAGCCUAGGAGAUUGC GCGCGGG 5'



as a function of the number of bases and the helical periodicity of the duplex in between the bulges, as indicated schematically in *b*. The double-bulged species were electrophoresed in 15% polyacrylamide, and the resulting autoradiographs presented for RNA (*c*) and RNA-DNA (*d*). The modulation of mobility with spacing is clear. In *e* and *f*, the mobilities of the RNA and RNA-DNA species respectively are plotted against the spacing between the two bulges, for the species around the minimum mobility, and fitted to a polynomial. It was found that the fit was improved by omission of the point corresponding to a spacing of 13 bp ($R^2 = 1.00$, as opposed to 0.97), presumably because of local sequence effects. The spacing giving the minimum mobility was calculated from the first derivative, and hence the helical periodicities of RNA and RNA-DNA were calculated to be 11.3 ± 0.1 and 10.9 ± 0.1 bp per turn respectively.



To investigate the effect of bulge size and sequence on the electrophoretic migration anomaly, we constructed 40-base pair oligoribonucleotide duplexes containing 0, 1, 3, 5 or 7 bulged adenine or uracil bases located at the centre of the fragment. The gel electrophoresis behaviour of these molecules in both RNA and RNA-DNA duplexes is shown in Fig. 2. The bulges were located in a constant sequence context, between G and U bases on the RNA strand. For both types of duplex, the extent of electrophoretic retardation correlated with the number of bulged bases, and was greater for the adenine than for the uracil bulges for a particular size of bulge. The sequence-dependent difference in the gel migration of the bulged molecules was most pronounced in the RNA helices. These results are most simply interpreted in terms of the introduction of a kink, which bends the helix axis by an angle that depends on the number and type of unpaired bases.

To establish whether the RNA bulges introduced a bend or kink into the duplex, RNA helices were synthesized to contain an A₅ bulge at either 5, 10, 14, 20 (centre), 25 or 34 base pairs (bp) from the left-hand end of the helix. This experiment is akin to the permutation analysis of DNA bending²⁸. The relative migration of these 40-bp duplexes is analysed in Fig. 3: maximum retardation occurs when the bulge is centrally located, indicating that the bulge is the cause of the reduced migration. The retardation is not simply a size effect due to the bulged bases, because a 45-bp RNA helix containing a central (A·U)₅ duplex in place of the bulge migrates faster than the species containing an A₅ bulge at the equivalent position. It is therefore likely that the RNA bulges introduce a precise geometry into the duplex, probably analogous to the highly defined kink present in DNA helices²³⁻²⁵.

If the effect of the bulge is to kink the RNA duplex in a precise way, then the overall shape of a molecule containing two such kinks should depend on the phasing between them, generating a dihedral angle between the two end portions of the molecule as shown in Fig. 4. As the phasing will depend on the distance between the two bulges, and the periodicity of the double-stranded RNA or RNA-DNA between them, we may use this to provide an estimate of the helical periodicity of these duplexes. We constructed a series of 60-bp isomeric RNA duplexes in which each fragment contained two A₅ bulges, and the relative separation of the two bulges was varied by 7, 9, 10, 11, 12, 13, 14, 15, 17 or 19 bp. The relative migration on polyacrylamide gels of these species, both as RNA and RNA-DNA, is shown in Fig. 4. For both species, there is the expected variation in mobility with spacing, which is roughly sinusoidal, with a minimum mobility corresponding to ~11 bp between bulges. As the spacing is made significantly longer or shorter than this, the length of the ends of the molecules changes, which should also affect gel mobility and lead to a distortion of perfect sinusoidal dependence. The mobilities of the species around the minimum of electrophoretic mobility were fitted to polynomial functions (Fig. 4), and from the first derivatives we obtained measurements of the helical periodicities for double-stranded RNA of 11.3 ± 0.1 bp per turn, and for RNA-DNA of 10.9 ± 0.1 bp per turn. The value for RNA is typical of an A helix¹¹, whereas that of the RNA-DNA is intermediate between RNA and the known periodicity of DNA (10.6 bp per turn^{29,30}), which is consistent with its intermediate mobility as a simple duplex. Any small errors in our measurements are probably due to sequence effects (the sequences were chosen as 'random' sequences, but at this level nothing is truly random) and to end-effects at the bulges themselves. The helical periodicity of a short RNA-DNA hybrid helix has been estimated as 10.9 bp per turn in the crystal¹⁸, but to our knowledge this is the first measurement of the helical periodicity of either RNA or RNA-DNA in solution. Our results, therefore, are consistent with the formation of a defined kink in double-stranded RNA resulting from the presence of bulged bases. Bulge-induced kinks in these molecules are significant in

the tertiary folding of RNA and as potential recognition sites for RNA-binding proteins. □

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1. Pleij, C. W. A., Rietveld, K. & Bosch, L. *Nucleic Acids Res.* **13**, 1717-1731 (1985).
2. Puglisi, J. D., Wyatt, J. R. & Tinoco Jr, I. *Nature* **321**, 283-286 (1988).
3. Delarue, M. & Moras, D. *Nucleic Acids and Molecular Biology* Vol. 3 (eds. Eckstein, F. & Lilley, D. M. J.), 182-196 (Springer-Verlag, Berlin and Heidelberg, 1989).
4. Wyatt, J. R., Puglisi, J. D. & Tinoco Jr, I. *BioEssays* **11**, 100-106 (1989).
5. Cech, T. R. *et al. Proc. natn. Acad. Sci. U.S.A.* **80**, 3903-3907 (1983).
6. Inoue, T. & Cech, T. R. *Proc. natn. Acad. Sci. U.S.A.* **82**, 648-652 (1985).
7. Uhlenbeck, O. C. *Nature* **328**, 596-600 (1987).
8. Haseloff, J. & Gerlach, W. L. *Nature* **334**, 585-591 (1988).
9. Baudin, F. & Romanuk, P. J. *Nucleic Acids Res.* **17**, 2043-2056 (1989).
10. Wu, H.-N. & Uhlenbeck, O. C. *Biochemistry* **26**, 8221-8227 (1987).
11. Arnott, S., Fuller, W., Hodgson, A. & Prutton, I. *Nature* **220**, 561-564 (1968).
12. Arnott, S., Chandrasekharan, R., Millane, R. P. & Park, H.-S. *J. molec. Biol.* **188**, 631-640 (1986).
13. Quigley, G. J. *et al. Proc. natn. Acad. Sci. U.S.A.* **72**, 4866-4870 (1975).
14. Jack, A., Ladner, J. E. & Klug, A. *J. molec. Biol.* **108**, 619-649 (1976).
15. Moras, D. *et al. Nature* **288**, 669-674 (1980).
16. Shevitz, R. W. *et al. Nature* **278**, 188-190 (1979).
17. Woo, N. H., Roe, B. A. & Rich, A. *Nature* **286**, 346-351 (1980).
18. Wang, A. H.-J. *et al. Nature* **299**, 601-604 (1982).
19. Dock-Bregeon, A. C. *et al. Nature* **335**, 375-378 (1988).
20. Varani, G., Wimberley, B. & Tinoco, I. *Biochemistry* **28**, 7760-7772 (1989).
21. Chou, S.-H., Flynn, P. & Reid, B. *Biochemistry* **28**, 2422-2435 (1989).
22. Zhang, P. & Moore, P. B. *Biochemistry* **28**, 4607-4615 (1989).
23. Hsieh, C.-H. & Griffith, J. D. *Proc. natn. Acad. Sci. U.S.A.* **86**, 4833-4837 (1989).
24. Bhattacharyya, A. & Lilley, D. M. J. *Nucleic Acids Res.* **17**, 6821-6840 (1989).
25. Rice, J. A. & Crothers, D. E. *Biochemistry* **28**, 4512-4516 (1989).
26. Woodson, S. A. & Crothers, D. M. *Biochemistry* **27**, 3130-3141 (1988).
27. Chou, S.-H., Flynn, P. & Reid, B. *Biochemistry* **28**, 2435-2443 (1989).
28. Wu, H.-M. & Crothers, D. E. *Nature* **308**, 509-513 (1984).
29. Rhodes, D. & Klug, A. *Nature* **292**, 378-380 (1981).
30. Peck, L. J. & Wang, J. C. *Nature* **292**, 375-378 (1981).
31. Beaucage, S. L. & Caruthers, M. H. *Tetrahedron Lett.* **22**, 1859-1862 (1981).
32. Sinha, N. D., Biernat, J., McManus, J. & Köster, H. *Nucleic Acids Res.* **12**, 4539-4557 (1984).
33. Milligan, J. F., Groebe, D. R., Witherell, G. & Uhlenbeck, O. C. *Nucleic Acids Res.* **21**, 8783-8798 (1987).

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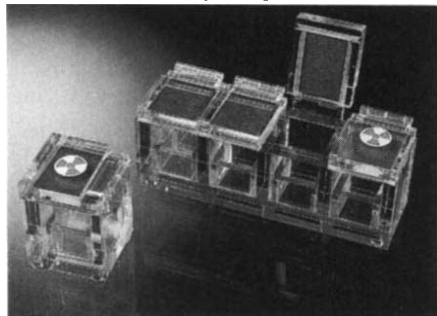
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York Biologicals is offering a complete range of **human growth factors** produced by recombinant DNA technology (*Reader Service No. 101*). The complete line includes epidermal growth factor, insulin-like growth factors I and II, human transforming growth factor- α , human growth hormone, and platelet-derived growth factor α -a and α -b. All of the growth factors have a purity of greater than 99 per cent, and are fully active biologically compared to native forms, says York. They are supplied as a lyophilized white powder that is soluble in water or most aqueous buffered solutions. Prices range between \$395 and \$7,200 (US) for 1 mg, although human growth hormone is sold only in 50-mg quantities for \$4,999 (US). The other growth factors are also available in 10-, 50-, and 100-mg quantities.

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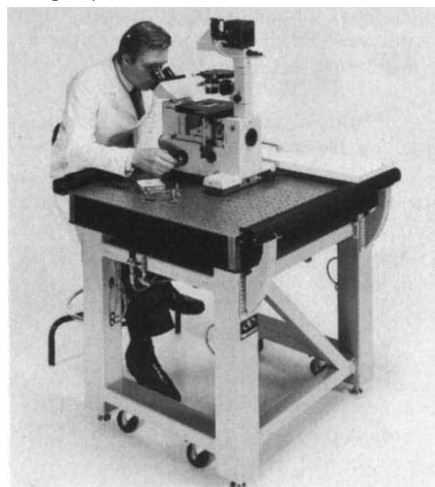
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Monitor beta radiation levels and detect contamination in the laboratory with the new Model 900-E Mini Monitor from Research Products International (*Reader Service No. 103*). The \$485 (US) Model 900-E's thin-end window G-M survey meter can be used to detect isotopes such as ^{32}P , ^{14}C , ^{35}S , ^{45}Ca and ^{131}I . Contamination levels as low as $8^{-4} \mu\text{Ci cm}^{-2}$ for 'hard' beta emitters and $10^{-4} \mu\text{Ci cm}^{-2}$ for soft emitters can be measured. Additional design features include a visual display of readings from 0 to 2,000 counts per second, a built-in adjustable warning alarm, integrated circuitry, a rechargeable NiCad pack, or an optional a.c. power supply.

Under the microscope

For laser and optical research applications that demand a **vibration-free platform**, Newport Bio-Instruments has introduced the VW Series of Vibration-Isolated Workstations (*Reader Service No. 104*). The VW Series provides, says the company, a worksurface that is free of



Newport provides vibration-free workstations to suit all lab needs.

both vertical and horizontal vibrations, which is important for laboratories situated in multistorey buildings. The workstation is offered in six different sizes ranging from 30 $\times$ 36 inches to 36 $\times$ 60 inches, with a price range of \$2,200–3,300 (US). Newport offers up to five work-surfaces, including stainless steel, with and without mounting holes, and plastic laminates. Newport has built in several design features to provide comfortable all-day operation, including a lower

30-inch worksurface height, optional, adjustable padded armrests and shelves for equipment.

Magnifications of up to 200,000 $\times$ and a large eucentric stage for holding specimens up to 125 mm in diameter are features of the JSM-5300 **scanning electron microscope** from Jeol (*Reader service No. 105*). The JSM-5300 has many automatic control, memory and data display functions for obtaining clear images or



JSM-5300: Jeol's user-friendly SEM.

photographs, which allow the instrument to be used by experienced and inexperienced persons alike, says Jeol. Operators can set the magnification in 25 steps to between 15 and 200,000 $\times$, with a resolution of 4.5 nm. The JSM-5300's stigmator memory stores values of astigmatism at any accelerating voltage and working distance, eliminating lengthy astigmatism correction procedures. An optional feature is the image framestore system which provides flicker-free stored images. The display can be subdivided into four areas to allow simultaneous comparative observations to be made. An energy dispersive X-ray spectrometer can be interfaced with the SEM for elemental analysis of surfaces.

Diagnostic AIDS

Speedscreen_{HIV} is the new **HIV-1 and HIV-2 strip-based assay** from British Biotechnology which was developed from basic research carried out at Oxford University (*Reader Service No. 106*). The assay uses synthetic antigens from HIV-1 and HIV-2, which are in a polyvalent form and bound to synthetic strips, to detect antibodies to p24, p27 (nef), gp41 (HIV-1) and gp36 (HIV-2). According to BBT, Speedscreen_{HIV} can be used to distinguish and differentiate between HIV-1 and HIV-2 on a single strip and may have uses in monitoring disease progression via changes in p24 and p27 antibody levels. In

a procedure that takes less than 45 minutes, samples are incubated with each strip, labelled with a second antibody, and the results determined either by eye or quantified by densitometry. Speed-screen_{HIV} kits, available only in the UK at present, contain enough reagents for 24 assays.

Wellcome Diagnostics has introduced the Wellcozyme kit which detects antibodies to HIV-1 and HIV-2 (Reader Service No. 107). The kit is based on recombinant HIV-1 core and envelope fusion protein, an ultrapure synthetic HIV-2 envelope peptide, and an amplified enzyme detection system. Using enzyme-labelled antigen sandwich technology, test samples and control sera are incubated in antigen-coated microwells. Conjugate, a mixture of the same antigens but labelled



Wellcome's HIV-1 and 2 detection kit.

with alkaline phosphatase, is added, and this binds to any specific antibody already bound to antigen on the microwell. The addition of an amplifying substrate system for alkaline phosphatase produces a coloured product which is read spectrophotometrically at 482 nm. The Wellcozyme kit contains an antigen-coated microwell plate, control sera, and all the necessary reagents to perform 96 tests.

INCSTAR has developed a radioimmuno assay system — ZDV-Trac¹²⁵I — for assessing the levels of AZT and G-AZT in serum samples (Reader Service No. 108). The ZDV-Trac¹²⁵I kit is a competitive direct equilibrium assay that uses a ZDV (AZT) derivative for both standards and tracer, with no serum extraction necessary. Standards, samples and controls are incubated for two hours at 25 °C with an iodinated tracer and rabbit anti-AZT antibody, after which there is a further incubation with a goat anti-rabbit precipitating complex. Bound and unbound tracer is separated by centrifugation and the pellet counted in a gamma counter. The counts obtained are inversely proportional to the amount of AZT in the sample. G-AZT determinations use the same methodology except the G-AZT must be enzymatically converted to AZT before performing the RIA. Kits come with sufficient antibody, radioactive tracer, precipitating complex, sample diluent, controls and standards to assay 130 tubes.

Cell science

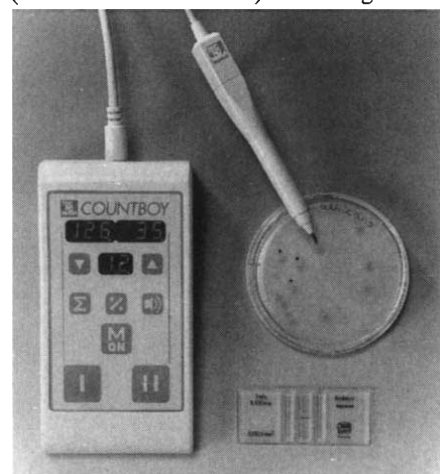
Vitrodyne is a system that has been developed by Liveco Biomechanical Instruments for determining the *in vitro* mechanical properties of living cells, tissues and non-biological materials (Reader Service No. 109). At the heart of the system is a



Vitrodyne takes the stress and strain out of data collection.

forcing frame which holds the sample suspended in a glass culture vessel. Vitrodyne can be programmed to execute a static or dynamic force profile. Forces can be applied to individual cells when they are embedded in a 3-dimensional matrix or adhered to an elastic substrate. The operator inputs parameters for force, waveform, amplitude, period and polarity into the computerized controller. Feedback circuitry allows the operator to control loads with a resolution as low as 10-mg force. Using the new add-on module (\$2,500 (US)), Vitrodyne provides the simultaneous output of both stress and strain data. The basic \$9,800 (US) set up includes a computerized controller, forcing frame, culture glassware, signal transducer module, thermal printer and specimen interface pieces.

Countboy is the latest biological gadget from Tecnomara of Germany, designed to make the counting of cells and colonies quicker and more accurate (Reader Service No. 110). Counting can be

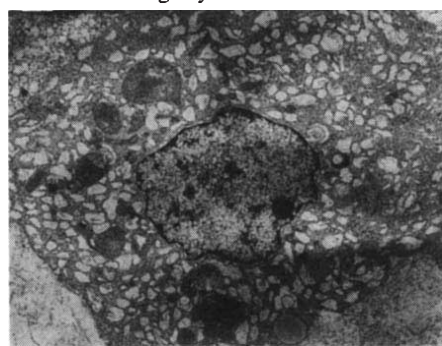


Countboy: the colony counter.

performed manually with the aid of a counting pen, or by operating a foot-switch. Two three-position LED channels allow the user to simultaneously and separately count live and dead cells. The

memory can store all single and double channel counts in a total of 48 memory positions and recall them through the station finder keys. The DM685 (FRG) Countboy can calculate percentages and averages and can be interfaced to a printer or a PC. For complete portability, the instrument can be run off its own rechargeable power pack.

The collaborative efforts of Epicentre Technologies and the University of Wisconsin have isolated Brefeldin A from the fungus *Eupenicillium brefeldianum* (Reader Service No. 111). Brefeldin A appears specifically to block protein translocation from the endoplasmic reticulum (ER) to the Golgi apparatus without, says Epicentre, affecting endocytosis, lysosome function, endosome acidification or the trans-Golgi system. The effects are



Brefeldin A for cellular studies.

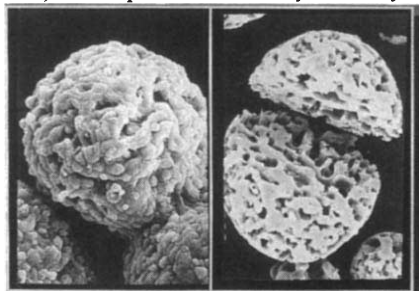
reversible and specific to the intracellular trafficking of proteins. In concentrations as low as 40 ng ml⁻¹, Brefeldin A has been shown to cause the disassembly of the Golgi complex and swelling of the ER. Brefeldin A costs \$85 (US) for 1 mg and is supplied as a white crystalline solid with a purity of greater than 99 per cent.

The new fully programmable cell harvester from Skatron, Inc. can automatically harvest a 96-well microplate or macrowell plate in one step (Reader Service No. 112). The unique block design and upward fluid flow for harvesting from underneath the filtermat, utilizes, says Skatron, the entire filter disc area and eliminates the 'gravity effect' common to other harvesting systems. Operators can select up to six washes from three inlet ports, preset the length of wash and dry sequences to minimize the amount of waste disposal, and store up to ten harvesting protocols. Additional design features include an internal pressure pump, dual outlets for the separation of 'hot' and 'cold' waste, and a 16 × 16-inch filtermat that is compatible with the LKB Wallac Betaplate counter. The Skatron cell harvester is priced at \$11,500 (US).

The horseradish peroxidase substrate 3-amino-9-ethylcarbazole is now available in tablet form from Sigma (Reader Service No. 113). The substrate can be used for

immunoblotting and immunohistological staining procedures and produces a red insoluble end product. Each tablet contains 20 mg of substrate. Tablets are supplied individually wrapped in foil packets in quantities of 50 and 100 tablets, costing \$49.50 and \$77.00 (US), respectively.

Percell Biolytica of Sweden has announced a new line of **macroporous gelatin microcarriers** (Reader Service No. 114). Cultispher-G is an enzymatically de-



Percell's macroporous microcarriers.

gradable matrix that is cross-linked for stability. It provides a large surface area, high attachment efficiency and is suitable for the growth of cell lines such as CHO-K1, R-CHO, MDCK, Vero, BHK-21, V79, L929, F9 and Hela, says Percell. Cultispher-GL has twice the pore size of Cultispher-G and is suitable for cell lines with a larger cell size. For researchers needing a microcarrier with a high density, Percell has developed Cultispher-GD, which incorporates a titanium compound into the matrix. Finally, Cultispher-GLD, a hybrid between the -GL and -GD types, combines the features of large pore size with high density. The Cultispher-G, -GL, -GD and -GLD microcarriers cost \$91, \$91, \$73 and \$73 (US), respectively, for a 10-g quantity.

Probes and primers

Lagan, Inc. is making available, in kit form, a series of **probes** that could have applications in mapping the human genome and that of higher primates (Reader Service No. 115). Specifically, the probes individually identify different sequences that are repeated 300 to 700 times throughout the human haploid genome and, consequently, can be used in a procedure (outlined in the kit) to identify contigs useful in the rapid mapping of genomic DNA. The kit includes six different 1- μ g probes and is suitable for cloned genomic DNAs in YACS, phages, cosmids and other vectors. Lagan says the kit, priced at \$300 (US), will be available after 1 March.

Gibco/BRL's expanded selection of **sequencing primers** now includes the M13/pUC Forward 23-Base Sequencing Primer (Reader Service No. 116). This extended-length primer is, says Gibco/BRL useful for high-temperature (72 °C) Taq DNA

polymerase-based sequence analysis of inserts in any of the M13mp or pUC vectors. The synthetic oligodeoxyribonucleotide is designed to hybridize to the lac α -peptide fragment sequence on the 3' side of the multiple cloning site, after which standard chain termination reactions can be performed. The 23-base length primer — 5'-CCCAGTCACGACGTTGTAAAACG-3' — remains annealed to the template during the initial part of the extension reaction, which is important when using Taq DNA polymerase at high temperatures, says the company.

Of proteins and DNA

Isonet **ion-exchange cartridges** provide a rapid and simple means of purifying proteins and are an alternative to shallow-bed ion-exchange columns, says Kinetek Systems (Reader Service No. 117). So far, two types have been made available: Isonet-S2 SP with sulfopropyl charge groups (strong cation exchanger) and Isonet-D2 DEAE with diethylaminoethyl charge groups (weak anion exchanger). As solute crosses the microporous hollow fibre walls, proteins with charges opposite to those of the functional group bind to the ion-exchange groups present on the interior surfaces of the pores. Isonet cartridges provide, says Kinetek, a capacity of about 2 mg, high flow rates, and protein that is greater than 95 per cent pure concentrated in minimal elution volumes. The cartridges are \$50 (US) for a pack of five and are colour-coded to indicate the functional group.

For concentrating, purifying and desalting small-volume samples of 3.5 ml or less which contain peptides, proteins, enzymes, viruses, antibodies and other macromolecules, Filtron Technology recommends the new **MICROSEP centrifugal concentrator** (Reader Service No. 118). MICROSEP incorporates Filtron's



MICROSEP — the multipurpose concentrator.

Omega series low protein binding ultra-filtration membrane, which is available with molecular weight cutoffs of 3 K, 10 K, 30 K, 100 K, 300 K and 1,000 K. The sample is loaded into the device, spun, and the concentrate extracted with a pipette tip, eliminating, says Filtron, the

need for backspinning when recovering the sample. MICROSEP is colour-coded for easy identification of molecular weight cutoff, and is not fitted with an O-ring so as to minimize leachables and broaden the chemical compatibility of the device.

Glen Research has brought out a line of **Poly-Pak cartridges for purifying DNA** which contain a polystyrene resin designed to overcome the problems associated with silica gel-based reverse-phase cartridges (Reader Service No. 119). Up to 30 OD units of a synthetic oligo can be purified in less than 15 minutes, says Glen, as there is no need to evaporate the ammonium hydroxide deprotection solution, wash volumes can total less than 15 ml, and the product can be eluted in four drops of acetonitrile. The cartridges come with luer-lock fittings for attachment to syringes or a vacuum manifold. In addition to the purification of standard oligos, Poly-Pak cartridges can be used to purify modified and labelled oligos. The cartridges are \$8 (US) each. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. To obtain further details, use the reader service card bound inside the journal. Prices quoted are sometimes nominal, and apply only within the country indicated.

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Reader Service No.50

What effect will student loans have?

Richard Pearson

The decision of school-leavers in the United Kingdom to go on to higher education in the next few years will be affected by the introduction of student loans.

THE supply of qualified scientists and technologists in the 1990s will be greatly affected by the impact of the introduction in the United Kingdom of student loans on student entry to higher education. This has been a matter of controversy and debate throughout the 1980s and the banks recently withdrew their offer to administer the scheme when students threatened to withdraw their accounts.

Under the new scheme, the maintenance grant will be frozen at the present £2,425 (for a student living away from home outside London) and a loan worth £420 will be made available at zero real rate of interest. The loan will be increased with the cost of living until it equals the maintenance grant, probably around the end of the century. At the same time, students will no longer be eligible for housing and other state benefits.

The long-term aim is to reduce public expenditure on student support, which now stands at £800 million, to introduce more market forces into student choice and to encourage wider participation in higher education at no extra cost. In reality, however, the costs of this scheme will be so high that public expenditure will rise for the rest of this century and will begin to be paid off only by the year 2013 at the earliest. Some critics argue that it will be even longer before the scheme will show any 'profit'.

As well as the debate about the cost and administration of loans, there is concern that their introduction will turn away students from higher education, especially those from poorer homes and from under-represented groups.

So how are students supported financially now? In 1988–89, the average young student in higher education received a total income from grants and their parents

of £2,461 (Fig. 1)¹. This was made up of an award of £1,159, an average contribution from parents of slightly more, £1,223 and £79 from other bursaries and employers. The grant itself is means-tested — grants are reduced once parents' net income exceeds £10,000 a year. Last year, only 23 per cent of students received the full grant, while one in three students received no award. For 46 per cent, the amount they received from their parents was above that assessed, 10 per cent receiving more than £600 over the assessment, while 31 per cent received less than the assessment with 10 per cent receiving at least £600 below the assessed amount. Four per cent were sponsored by an employer.

Vacation work is increasingly being seen as an important way of gaining relevant work experience and this provided a further £102 in the short vacations and £502 in the long vacation. The main differentiation in earnings in the long vacation was that those in colleges earned the least (they were most likely to be on arts courses) as did women and those living outside London. Other term-time work added £85, while benefits added £84, loans and savings £232 and other sources £202, to give a total income of £3,166 for the academic year plus vacation earnings. While many of these incomes showed little difference between social classes, students from the middle class homes received by far the most in the form of gifts and other income with the result that their total income, at £3,607, was more than £600 higher than for those from the lower social classes.

The chief determinant of expenditure was whether the student was living away from home. Otherwise, those with the highest incomes were spending more, particularly on entertainment and, interest-

ingly, course expenses. These averaged £221 for all students but rose to £293 for the top social class. They were highest for those studying medicine (£392) and art (£297) and lowest in business administration (£515) and biology (£512).

The introduction of the student loan will also be accompanied by the freezing and, effectively, reduction in the real size of the grant, which has fallen by 25 per cent in real terms since 1962. Those most affected will be those on the largest grants who are from the lower income groups and social classes.

The big issue is whether this group, still significantly under-represented in higher education (see *Nature* 342, 102; 1989), will be encouraged or discouraged to enter higher education by the introduction of loans.

A separate report on the attitudes of young people aged 15–19 suggests that in this age group more of those from middle-class homes intend to take degrees (74 per cent) than do those from other social classes (60 per cent)². For nearly half of the latter group, financial considerations are important when deciding whether to go on to higher education whereas they are important to only one in six of those in the middle classes. One in four of the students said that loans would affect their plans, this proportion rising to more than a third among social classes III and IV, although only one in six of the latter group said they would be less likely to go to higher education. Only one in twenty in social classes I and II thought that way. A far higher proportion in classes III and IV said they hoped to get a job where the employer would pay for them to go to college than was the case among classes I and II, who were more likely to turn to their parents for support. In terms of choice, of course, few would seek to study a different subject, but a significant minority said they would choose a course nearer home.

The introduction of loans will remain controversial for some years to come, and we should know the extent to which it acts as a deterrent to entry to higher education, particularly among the lower social classes, within a couple of years. □

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1. Student Income and Expenditure (RSL, 1989).
2. Opportunity Lost (NUS, 1989).

Sources of student income for the 1988/89 academic year, excluding the long vacation. From ref. 1.

